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MOLECULAR SUBCLASSIFICATION, STEM CELL MARKERS AND GROWTH REGULATORY PATHWAYS IN GLIOMAS

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ABSTRACT

Glioblastoma is the most common and aggressive primary brain tumor in adults. Median survival is no more than 15 months, even with combined treatment regimens. The lack of accurate pre-clinical model systems has limited the development of new treatment options for this deadly disease.

In the first study we set out with the goal of creating improved research models for studying the disease. To this end we generated a glioblastoma patient derived cell line (PDCL) library. PDCLs grown under neural stem cell conditions functionally isolate glioblastoma cells with stem-like properties. In keeping with their cancer stem cell like nature PDCLs readily formed patient derived xenografts (PDXs) when transplanted *in vivo*, recapitulating histological features and maintaining genetic/protein profiles of founding tumors. Expression profiling of PDCLs revealed 4 *in vitro* subclasses differentially associated with WNT/B-catenin, TGF-B, BMP, Interferon, Notch and p53 signaling. Applying these new *in vitro* PDCL-classes to glioblastoma patient data generated clinically relevant subclasses with differences in age and survival. Hence, new PDCL models represent the original disease including aspects such as genomics/proteomics, intratumoral heterogeneity and cancer stem cell biology. In addition to functional isolation of stem-like cells using PDCL methods there have been several proteins proposed to distinguish cancer stem cells (CSCs). The most popular such protein is Prominin-1 (PROM1/CD133). In the second study we investigated Prominin-1 expression in the developing CNS and glioblastoma. Prominin-1 is expressed in the ventricular zone of the embryonic brain and postnatally shifts to a pattern of distributed cells. The adult murine *Prom1*$^{+}$ cell population mainly consists of Olig2^{+} slow cycling glial cells. Further, we established that *Prom1* is independent of Olig2. Human normal brain PROM1 is associated with GFAP and SOX2. In glioblastoma PDCLs PROM1^{+} cells can express GFAP, SOX2 and OLIG2. PROM1 patterns are very heterogeneous across PDCLs and patient samples revealed that high *PROM1* expression is less common in *IDH1* mutant samples. In the third and fourth studies we investigated the possible functions of NPM1 in glioblastoma. NPM1 is a nucleolar chaperone protein highly upregulated in glioblastoma tissue samples and cell lines. We showed that NPM1 regulated the morphology of the nucleolus. This is likely due to NPM1 interacting with proteins implicated in the organization of chromatin such as HP1γ, H1.5 and H3. We also found that NPM1/DNMT3A co-depletion induced rDNA transcription and nucleolar normalization. Although DNMT3A loss upregulated rDNA transcription on its own depletion of NPM1 increased this effect suggesting NPM1 loss creates a permissive environment for further epigenetic change. NPM1 depletion sensitized glioblastoma cell lines to chemotherapy and further insults to chromatin stability such as the co-depletion of H1.5, implying NPM1 as a novel drug target.

This thesis characterizes a glioblastoma PDCL library, illustrating the benefits of these new research models and addresses the complex expression of Prominin-1 and function of NPM1 with regards to molecular subclasses and growth regulatory pathways in glioblastoma, respectively.

LIST OF SCIENTIFIC PAPERS

I. Integrative pathogenomic analysis of a glioblastoma cell line library reveals novel tumor cell intrinsic subclass distinctions

Karl Holmberg Olausson, Shakti Ramkissoon, Matthew A. Theisen, Ahmed Idbaih, Cecile L. Maire, Justin Craig, Margot Burns, Azra H. Ligon, Monica Nistér and Keith L. Ligon.
Manuscript.

II. Prominin-1 (CD133) defines both stem and non-stem cell populations in CNS development and gliomas

Karl Holmberg Olausson[*], Cecile L. Maire[*], Sam Haidar, Jason Ling, Emily Learner, Monica Nistér and Keith L. Ligon.
PLoS One. 2014 Sep 3;9(9):e106694.
[*]These authors contributed equally to this work.

III. Loss of nucleolar histone chaperone NPM1 triggers rearrangement of heterochromatin and synergizes with a deficiency in DNA methyltransferase DNMT3A to drive ribosomal DNA transcription

Karl Holmberg Olausson, Monica Nistér and Mikael S. Lindström.
J Biol Chem. 2014 Dec 12;289(50):34601-19.

IV. Expression and cellular localization patterns of the histone chaperone NPM1/nucleophosmin in glioma

Karl Holmberg Olausson, Tamador Elsir, Monica Nistér and Mikael S. Lindström.
Manuscript.

CONTENTS

LIST OF ABBREVIATIONS

5-FU	5-Fluorouracil
5-TM	5-transmembrane
ARF	alternative reading frame protein (p14/p19Arf)
ATRX	alpha thalassemia/mental retardation syndrom X-linked
bFGF	basic fibroblast growth factor
BMP	bone morphogenetic protein
BTC	betacellulin
CC3	cleaved caspase 3
CDK4/6	cyclin-dependant kinase 4/6
CDKN2A/B/C	cyclin-dependant kinase inhibitor 2A/B/C
chr	chromosome
CNS	central nervous system
CSC	cancer stem cell
CTCF	CCCTC-binding factor
DCX	doublecortin
DFC	dense fibrillar component
DNMT1/3A	DNA (cytosine-5)-methyltransferase 1/3 alpha
EGF	epidermal growth factor
EGFR	epidermal growth factor receptor
EGFRvIII	epidermal growth factor receptor variant III
FC	fibrillar component
G-CIMP	glioma CpG island methylator phenotype
GBM	glioblastoma
GC	granular component
GEMM	genetically engineered mouse model
GFAP	glial fibrillary acidic protein
GNPC	granular neuron precursor cell
HDAC	histone deacetylase

HP1	heterochromatin protein 1
hTERT	human telomerase reverse transcriptase
IDH1	isocitrate dehydrogenase 1
IGF2	insulin-like growth factor 2
iSVZ	inner subventricular zone
kDa	kilodalton
KLF4	Kruppel-like factor 4
LOH	loss of heterozygosity
MAPK	mitogen-activated protein kinase
MDM2/4	mouse double minute 2/4
MEF	mouse embryonic fibroblasts
MGMT	O-6-methylguanine-DNA methyltransferase
MVP	microvascular proliferation
NF1	neurofibromin 1
NG2	chondroitin sulfate proteoglycan 4
NOD/SCID	non-obese diabetic/severe combined immunodeficiency
NSC	neural stem cell
OB	olfactory bulb
OLIG2	oligodendrocyte lineage transcription factor 2
oSVZ	outer subventricular zone
PDCL	patient derived cell line
PDGFRA	platelet-derived growth factor receptor, alpha
PDX	patient derived xenograft
PI3K	phosphoinositide-3-kinase
PIK3CA	phosphatidylionsitol-4,5-bisphosphate 3-kinase catalytic subunit alpha
PIK3R1	phosphoinositide-3-kinase, regulatory subunit 1
PTCHD	patched domain-containing protein
PTEN	phosphatase and tensin homolog
RA	retinoic acid
RB1	retinoblastoma 1

rDNA	ribosomal DNA
RMS	rostral migratory stream
RNA pol I	RNA polymerase I
RP	ribosomal protein
RTK	receptor tyrosine kinase
SGZ	subgranular zone
SHH	sonic hedgehog
SIRT1	sirtuin 1
snoRNP	small nucleolar ribonuclear protein
SOX2	SRY (sex determining region Y)-box 2
SUV39H1	suppressor of variegation 3-9 homolog 1
SVZ	subventricular zone
TCGA	the cancer genome atlas
TMZ	temozolomide
VZ	ventricular zone
WHO	World Health Organization
WNT	wingless

1 INTRODUCTION

Gliomas are tumors of the central nervous system (CNS) with glioblastoma (GBM) being the most common high-grade (grade IV) malignant glioma in adults (Louis et al. 2007). Glioblastoma has a terrible prognosis, median survival is not more than 12-15 months even with combined treatment regimens (Stupp et al. 2005; Louis et al. 2007; Van Meir et al. 2010). Chemotherapy (temozolomide based) and radiotherapy have insufficient effects on these tumors. Surgical treatment only offers temporary relief due to the highly infiltrative growth of the tumor cells and the negative effects of removing surrounding neural tissue, making it impossible to achieve complete tumor resection. Even when all of these methods are combined relapse is imminent. Not only do all patients relapse but there is also a trend of increased malignancy in the recurring tumor (Kang and Kang 2007; TCGA 2008; van Thuijl et al. 2015). The main problem is the fact that all tumor cells with the ability to regenerate the tumor are not removed/ablated by the treatments. These cells have been termed cancer stem cells (CSCs) and tumor initiating cells (TICs) with the glioma specific variants of the terms being glioma stem cells (GSCs) and glioma initiating cells (GICs) (Singh et al. 2003; Sakariassen et al. 2007; Griffero et al. 2009; Anido et al. 2010). For this thesis I will use the term cancer stem cells (CSCs). CSCs are described in several types of cancers and it is not a phenomenon restricted to CNS malignancies (Huang and Wicha 2008; Maitland and Collins 2008; Peacock and Watkins 2008). The cells of origin for gliomas are unknown. CSCs may be of particular interest to understand these origins, since it's believed that they could be the founding cells of the tumor. Finding the glioblastoma cell of origin in the normal tissue might give important clues on how to better tackle glioblastomas with new innovative treatments, whilst sparing normal brain structures.

Despite its rarity glioblastoma has during recent years been a leading model for the study of cancer, being the first disease selected to be characterized by The Cancer Genome Atlas (TCGA) due to its fast progression, limited treatment options and dismal survival rates. This has given us detailed knowledge about the genomic landscapes and molecular subclasses of glioblastoma (Verhaak et al. 2010; Brennan et al. 2013). These and other studies reveal very high degrees of tumor heterogeneity at genome, gene expression and protein levels including within and across patients. (Snuderl et al. 2011; Patel et al. 2014). Glioblastoma has also been one of the main cancers involved in the development of the re-emerging theory about CSCs, where PROM1 (Prominin-1/CD133) has been described as a prospective marker for identification and isolation of CSCs (Singh et al. 2003). The parallels drawn between the varying spectrum and degrees of differentiation in CSCs and neural stem cells (NSCs) are intriguing with possible application in tumor progression and tumor growth. DNA methylation and histone methylation/acetylation are other factors involved in regulating the cell state and dictating gene expression and consequently the differentiation of the cell (Jones and Baylin 2007). This controls the process of normal development and cell turnover in mature tissue as well as playing key roles in cancer (Bernstein et al. 2006). New methods for culturing normal CNS cells have also greatly helped the glioblastoma field, giving researchers a new generation of patient derived cell lines (PDCLs) able to maintain key

genetic alterations and mRNA/protein expression patterns found in the primary disease better than was possible in the last 50 years using conventional serum cultured cell lines (Hodgson et al. 2009; Wakimoto et al. 2011). These new cell models together with today's vast options in biological screens have opened the door for the more effective development of new targeted treatments. However, more work to develop methods, characterize the models and study the biology is needed to realize this potential.

1.1 NEURAL STEM AND PROGENITOR CELLS

With regards to gliomas, neural stem/progenitor cells hold great interest due to their possible roles as cells of origin, overlap in signaling pathways/transcriptional networks and phenotypic/proteomic similarities that may shed light on how to treat these tumors. Thus, NSC biology can be considered a foundation on which to base glioma/brain tumor biology. Following is a brief description of neural stem/progenitor cells with focus on cells and markers often implied in brain tumor biology.

1.1.1 Neural stem cells

There are a number of regions in the embryonic and adult brain that harbor neural stem cells (NSCs) in human and murine systems (Fuentealba et al. 2012; Florio and Huttner 2014; Lim and Alvarez-Buylla 2014). NSCs are multipotent and can generate all the three main cell types of the brain, i.e. neurons, astrocytes and oligodendrocytes. NSCs can be cultured *in vitro* where single NSCs proliferate and form spheres, known as neurospheres (Reynolds and Weiss 1992; Rietze and Reynolds 2006). No single marker protein can prospectively distinguish a NSC from other cells of the brain, but the proteins NESTIN, PROM1, GFAP, OLIG2, GFAPδ and SOX2 have emerged as useful markers (Uchida et al. 2000; Ellis et al. 2004; Roelofs et al. 2005; Menn et al. 2006; Ligon et al. 2007; van den Berge et al. 2010; Codega et al. 2014). Differentiated neural cell types can be identified with their own set of markers; oligodendrocytes can be identified by the markers CNPase and O4, astrocytes by GFAP and S100B whereas neurons are positive for TUJ1 and NeuN. Said markers are also used in differentiation assays of NSCs confirming multipotency (Uchida et al. 2000; Lee et al. 2005). Using several of these markers together can then allow further mapping of cell identities and differentiation stages.

During embryonic development there is an extensive number of stem/progenitor cells in the ventricular zone (VZ) of the brain and regions of the cerebellum/brain stem including the rhombic lip (Hartfuss et al. 2001). Stem/progenitor cells in the adult mouse brain are found in the subventricular zone (SVZ) of the lateral ventricular wall and the subgranular zone (SGZ) in the dentate gyrus of the hippocampus (Alvarez-Buylla and Lim 2004). Continuous waves of cell proliferation and differentiation can be observed in these areas (Lim et al. 1997; Lim and Alvarez-Buylla 1999; Alvarez-Buylla and Lim 2004). However there are also distributed progenitors throughout the adult CNS which in fact outnumber the SVZ niche stem/progenitor cells the most important of which is the Olig2 dependant $NG2^{+}$ progenitor cell (Ligon et al. 2006; Ligon et al. 2007; Canoll and Goldman 2008; Heinrich et al. 2014).

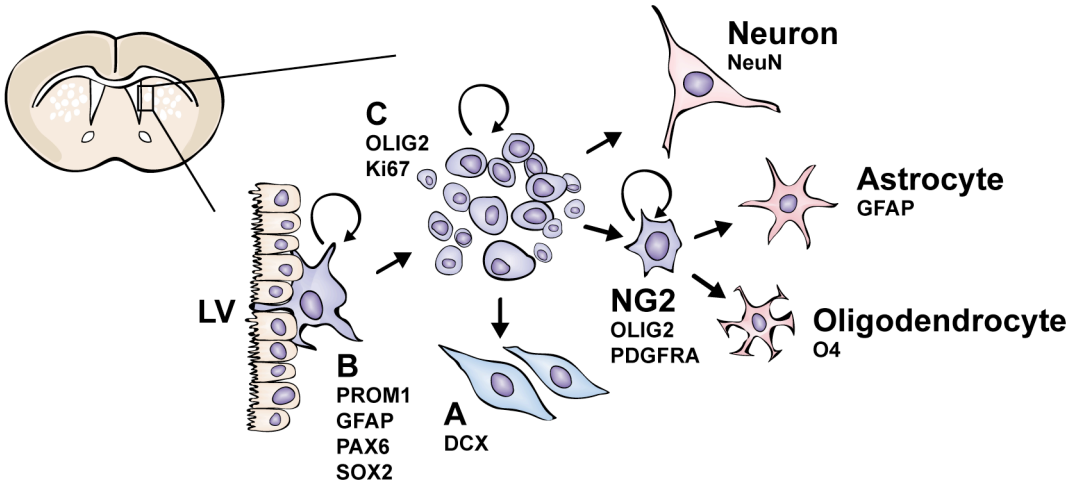

Figure 1. Neurogenesis in the adult SVZ, distributed progenitors and main cell types. Type B (NSCs), C (transit-amplifying progenitors), A (migrating neuroblasts) cells, NG2 stem/progenitor cells, Neurons, Oligodendrocytes and Astrocytes with associated markers. LV: lateral ventricle.

1.1.2 Neurogenesis in the subventricular zone

Subventricular zone (SVZ) neurogenesis in the adult brain has mainly been described in mice. The SVZ contains three important cell types; A, B and C cells (Fig. 1) (Doetsch et al. 1997; Mirzadeh et al. 2008). The B cells are described as the neural stem cells in the SVZ, these slowly dividing cells give rise to the more rapidly dividing C cells, transit-amplifying progenitors, which in turn give rise to the type A migrating neuroblasts (Jackson and Alvarez-Buylla 2008; Kriegstein and Alvarez-Buylla 2009). The SVZ population generates granule and periglomerular neurons for the olfactory bulb, it also responds to brain lesions resulting in the generation of astrocytes and oligodendrocytes suggesting further stem cell capabilities of the stem/progenitor cells from the SVZ (Romanko et al. 2004; Nait-Oumesmar et al. 2007). The SVZ is described as a stem cell niche, this concept implies the surrounding tissues influence on supporting/determining the cell fate of its resident cells (Shen et al. 2008; Fuentealba et al. 2012; Lim and Alvarez-Buylla 2014). This influence can be related to the role of the tumor microenvironment, which is proposed to sustain tumor cells and fuel tumor progression (Hanahan and Weinberg 2011; Quail and Joyce 2013).

1.1.2.1 The type B, C and A cells of the subventricular zone

The radial glia are the stem cells in the ventricular zone (VZ) of the developing brain, the adult B cells are radial glial-like cells and believed to have a radial glial origin (Doetsch et al. 1999; Heins et al. 2002; Merkle et al. 2004; Kriegstein and Alvarez-Buylla 2009). B cells can be identified by their Gfap, Pax6, Sox2, Gfapδ and Prom1 expression (Heins et al. 2002; Ellis et al. 2004; Roelofs et al. 2005; van den Berge et al. 2010; Codega et al. 2014). Quiescent B cells do not express Nestin and Egfr whereas these proteins are expressed when the B cells become active and re-enter the cell cycle (Codega et al. 2014; Khatri et al. 2014). The activation of B cells leads to the generation of $Olig2^{+}$ C cells (Ligon et al. 2006; Menn et al. 2006), which divide symmetrically approximately 3 times before transforming into Dcx^{+} type A neuroblasts that enter the rostral migratory stream (RMS) to the olfactory bulb (OB) (Fig.

1). Upon reaching the OB type A cells differentiate into olfactory neurons (Sawamoto et al. 2011; Ponti et al. 2013). Although the lateral ventricular wall SVZ is described as the main site of neurogenesis there are also neuroblasts generated from the dorsal wall of the lateral ventricle (Merkle et al. 2007; Ventura and Goldman 2007).

The SVZ has a very ordered structure where B, C and A cells are localized to specialized niches and there is an organized architecture to the cell relationships even within the niche environment. B cells extends a minute apical ending with a process situated in the ventricle and the cells basal process reaching into the SVZ onto blood vessels, and B cells are organized in a pinwheel structure together with ependymal cells (Mirzadeh et al. 2008). The apical process, suggested to be Prom1^{+}, is linked to sonic hedgehog (Shh) signaling and the CSF circulating in the ventricles has been shown to contain the growth factor ligands Shh, wingless (Wnt), bone morphogenetic proteins (Bmps), retinoic acid (RA), and insulin-like growth factor 2 (Igf2) (Mirzadeh et al. 2008; Wong and Reiter 2008; Lehtinen et al. 2011). These factors are believed to control the "stemness" and proliferation of B cells. The B cells contact with the vasculature is also proposed to help maintain their stem cell identity with Betacellulin (Btc) and EGF-like growth factor secreted from endothelial cells (Gomez-Gaviro et al. 2012). Furthermore, B cells are influenced by neighboring ependymal cells regulating Bmp signaling (Gajera et al. 2010). Another example is the feed back loop from newly generated neurons producing gamma-aminobutyric acid (Gaba) that inhibits B cell proliferation (Liu et al. 2005).

To add an extra order of complexity there is a differential region specific cell identity of the SVZ where NSCs from a certain region of the SVZ generate a specific type of neuron (Merkle et al. 2007; Merkle et al. 2013). Transplantation experiments suggest that this specificity is cell intrinsic and NSCs transplanted to a new region still maintain their specific identity. This implies that not all aspects of NSCs are determined by the SVZ stem cell niche/microenvironment. If this specificity is maintained in NSCs from their founding radial glia state it could be under epigenetic control mechanisms (Ladd-Acosta et al. 2007; Hirabayashi and Gotoh 2010; Lim and Alvarez-Buylla 2014).

1.1.2.2 The embryonic ventricular zone

Human embryonic brain, like the murine, contains a region in the ventricular wall with numerous radial glial and progenitor cells dividing in order to form all cells of the developing brain. However, the human brain has a larger neocortex, which is more cell dense compared to that of mice (Azevedo et al. 2009). The germinal cell layer of the embryonic brain consists of the VZ, inner SVZ (iSVZ) and the outer SVZ (oSVZ). The radial glia (apical radial glia) cells are located in the VZ with their processes reaching through the iSVZ and oSVZ out to the basal lamina of the brain. Both the iSVZ and oSVZ contain progenitor cells, the human brain needs to sustain the generation of a higher number of neural cells and is proposed to have a broader germinative niche. The iSVZ also contains sub apical progenitors and the processes of apical intermediate progenitors that have their cell soma in the VZ. Both the

iSVZ and oSVZ contain basal radial glia extending their processes to the basal lamina (Gotz and Huttner 2005; Florio and Huttner 2014).

1.1.2.3 The adult human subventricular zone

In the adult SVZ the differences are more apparent between mice and humans. In the adult human brain there is not a clear identification of the B, C and A cells and no RMS has been identified so far (Sanai et al. 2004; Sanai et al. 2011; Bergmann et al. 2012). While humans and mice both have an ependymal cell layer lining the lateral ventricles the human SVZ uniquely contains a structure named the astrocyte ribbon and has no clear germinal stem cell layer (Sanai et al. 2004; Quinones-Hinojosa et al. 2006). The astrocyte ribbon consists of astrocyte processes forming a structure located under the ependymal cells. The astrocyte cell bodies are then located further towards the striatum in a layer containing a great deal of vasculature. The function of this astrocyte cell layer is not yet understood and NSCs have not been definitively identified in this location and age. There is evidence that new neurons are generated in the striatum of the human brain. It is not known whether they originate from a certain stem cell niche, although the striatum is a structure adjacent to the SVZ (Ernst et al. 2014).

1.1.3 Neurogenesis in the subgranular zone of the hippocampus

The subgranular zone (SGZ) in the dentate gyrus of the hippocampus contains a neurogenic niche similar to that of the SVZ. Here subgranular zone $Sox2^+$ NSCs divide and give rise to progenitor cells that proliferate and mature into granule cell neurons that incorporate into the dentate gyrus granule cell layer (Alvarez-Buylla and Lim 2004; Piatti et al. 2006). $Sox2^+$ NSCs can both reside in the SGZ as $Gfap^+/Nestin^+$ radial-like and non-radial cells (Suh et al. 2007). They too originate from the radial glial cells in the VZ of the developing brain. However, SGZ NSCs predominately generate granular neurons and evidence from mouse models does not point to a large extent of glial cells being generated by $Sox2^+$ NSCs but they would appear to be able to generate astrocytes and oligodendrocytes *in vivo* (Suh et al. 2007; Jessberger et al. 2008). This selective differentiation is proposed as an effect of the SGZ niche promoting the formation of new neurons rather than glia. Upon exercise induced SGZ neurogenesis the pool of $Sox2^+$ NSCs will proliferate and differentiate in a type of steady state with the end result being a constant number of $Sox2^+$ NSCs but additional neurons (Suh et al. 2007). There is evidence that neurons are generated through out adulthood in the human hippocampus (Spalding et al. 2013).

1.1.4 Distributed stem/progenitor cells in the adult CNS

A little recognized fact is that the most abundant stem/progenitor cells in mouse and human brain are distributed cells existing throughout all regions of the CNS parenchyma. These glial stem/progenitors have been described as $NG2^+/A2B5^+/Pdgfra^+/Olig2^+$ cells (Ligon et al. 2006; Canoll and Goldman 2008), and found in large numbers in white and grey matter (Fig. 1). They are believed to have the capacity to form oligodendrocytes and astrocytes *in vivo*. If isolated and cultured *in vitro* they can form oligodendrocytes, astrocytes and neurons (Canoll

and Goldman 2008), but appear to be restricted in their lineage differentiation when *in vivo*. $NG2^+$ cells can however be reprogrammed into forming neurons *in vivo* by inducing *Sox2* expression following stab wound injury (Heinrich et al. 2014).

NG2 is a chondroitin sulfate proteoglycan expressed in stem/progenitor cells of the adult brain. $NG2^+$ cells have been shown to respond to mechanical injuries such as stab lesions (Buffo et al. 2005) and cryo-lesioning (Chen et al. 2008), as well as chemical lesions leading to demyelination (Islam et al. 2009). $NG2^+$ cell responses included proliferation and the differentiation into astrocytes and oligodendrocytes. $NG2^+$ cells are dependant on Olig2 function, Olig2 is a basic helix-loop-helix transcription factor expressed in oligodendrocytes and stem/progenitor cells (Ligon et al. 2006). Again our knowledge regarding this is based on murine systems there is however evidence that a few new oligodendrocytes are generated in the adult human brain (Yeung et al. 2014). $NG2^+$ cells are also abundant in human brain and accumulate to multiple sclerosis lesions (Chang et al. 2000). The reactive capabilities and sheer abundance of these cells could suggest them as likely cells of origin in gliomas.

1.1.5 Stem/progenitor cells in the cerebellum

The developing cerebellum has an influx of stem/progenitor cells from the VZ of the fourth ventricle and the rhombic lip (Hatten and Heintz 1995; Swartling et al. 2012). The wall of the fourth ventricle also contains radial glial cells that give rise to GABAergic progenitor cells and Bergmann glia (Edwards et al. 1990; Hoshino et al. 2005; Yamada et al. 2014). The GABAergic progenitors generate Purkinje neurons. The NSCs from the rhombic lip generate glutamatergic progenitors, which in turn give rise to granular progenitor cells finally differentiating into the granule neurons of the cerebellum (Ben-Arie et al. 1997; Fink et al. 2006; Hevner et al. 2006). In the adult cerebellum there is no evidence of an active germinal cell niche neither in the SVZ of the fourth ventricle nor in the remnants of the rhombic lip. There are however a number of $NG2^+$/$Olig2^+$ cells, representing the distributed glial stem/progenitor cell population as seen in the rest of the brain and distributed throughout the white and gray matter of the cerebellum (Dawson et al. 2003; Ligon et al. 2006).

1.2 GLIOMAS

Glioblastoma is the most malignant grade IV variant of the general brain tumor group termed gliomas. Glioma is a term used for several histological subtypes of tumors with morphological resemblance to normal glia in the brain, the main types being astrocytic and oligodedroglial tumors. The astrocytic tumors include; subependymal giant cell astrocytoma (grade I), pilocytic astrocytoma (grade I), pilomyxoid astrocytoma (grade II), diffuse astrocytoma (grade II), pleomorphic xanthoastrocytoma (grade II), anaplastic astrocytoma (grade III) and glioblastoma (classic, giant cell glioblastoma, gliosarcoma variants) (grade IV). Oligodendroglial tumors consist of oligodendroglioma (grade II) and anaplastic oligodendroglioma (grade III). Despite all being termed gliomas by their lineage and relation to glia, these tumors can not be considered as much related as different grades of tumors within the group of breast cancer grade I-IV and nor do they follow the same progression

pattern. Subependymal giant cell astrocytoma and pilocytic astrocytoma, both grade I, do normally not progress into higher grade tumors whereas diffuse astrocytoma (grade II) progresses to anaplastic astrocytoma (grade III) which subsequently can progress, but only rarely, to glioblastoma (termed secondary glioblastoma) (grade IV). Diffuse astrocytoma can also progress directly to glioblastoma (again referred to as secondary glioblastoma). Oligodendroglioma (grade II) progresses to anaplastic oligodendroglioma (grade III) but do not cross into the lineage of astrocytic tumors such as glioblastoma, although this is a somewhat controversial topic. Gliomas very rarely metastasize outside the brain however astrocytomas, oligodendrogliomas and glioblastomas are extremely infiltrative and their growth often ends up involving both brain hemispheres. Gliomas can display very heterogeneous histopathological features often shared among the different histological subtypes, in some cases making it impossible to visibly distinguish them from each other (Louis et al. 2007). However with new molecular markers as a complement to histology there have been several advances in glioma subclassification shedding light on the different biological events that are cornerstones in glioma development and progression. Here I will briefly describe the most common forms of gliomas based on the World Health Organization (WHO) 2007 Classification of Tumors of the Central Nervous System (Louis et al. 2007). Finally, I will provide a more in-depth review of glioblastoma, which is the main tumor type focused on in my thesis work.

1.2.1 Astrocytomas

1.2.1.1 Pilocytic astrocytoma

Pilocytic astrocytomas (WHO grade I) are defined as relatively circumscribed, slowly growing, often cystic astrocytomas, representing 5-6% of all gliomas. Pilocytic astrocytomas are the most common glioma in children and frequently localized to the cerebellum. The incidence rate is 0.37 per 100 000 population per year. They occur mainly in children and young adults with histological features of biphasic pattern with varying proportions of compacted bipolar cells associated with Rosenthal fibers and loose-textured multipolar cells, microcysts and eosinophilic granular bodies/hyaline droplets. Although being classed as a grade I neoplasm that even can regress spontaneously, some pilocytic astrocytomas can lead to medically challenging clinical symptoms including a wide spectrum of neurological deficits and may eventually be fatal if located in the hypothalamus or the brain stem. Pilocytic astrocytomas rarely progress to more malignant forms, but when this happens they often have features of glioblastoma and are referred to as anaplastic pilocytic astrocytoma (Louis et al. 2007). Pilocytic astrocytomas can be histologically very hard to distinguish from reactive gliosis (a non-neoplastic proliferation/reactive change in glial cells) and diffuse astrocytoma, but pilocytic astrocytomas often carry a fusion gene involving the kinase *BRAF* and relatively unknown gene *KIAA1549*, which serves to distinguish them from alternative histopathologic diagnoses (Jones et al. 2008).

1.2.1.2 Diffuse astrocytoma

Diffuse astrocytomas (WHO grade II) are diffusely infiltrating tumors typically affecting young adults, characterized by a high degree of cellular differentiation and slow growth. They have an incidence of 0.14 per 100 000 population per year. Diffuse astrocytomas are often located supratentorially and may follow a malignant progression to anaplastic astrocytoma followed by glioblastoma, or can directly progress to glioblastoma (termed secondary glioblastoma). Diffuse astrocytomas represent approximately 10-15% of astrocytic brain tumors and most commonly arise in the frontal and temporal lobes. Composed of well-differentiated fibrillary or gemistocytic neoplastic astrocytes with an often microcystic stroma. Diffuse astrocytomas display an increased cellularity compared to normal brain but there is little evidence of cell proliferation, mitotic activity assessed by Ki67/MIB-1 is less than 5%. Occasional nuclear atypia is common but tumor cells can be hard to distinguish from reactive astrocytes (Louis et al. 2007). Diffuse astrocytomas are characterized by several genetic hallmarks; *TP53* mutation has since long been associated with the disease, *IDH1* mutation (Yan et al. 2009), and *ATRX* mutation have in more recent years been found to be characteristic for the diagnose (Kannan et al. 2012; Liu et al. 2012). Diffuse astrocytomas have also been associated with the DNA methylation phenotype glioma-CpG island methylator phenotype (G-CIMP), which in turn can be associated with *MGMT* promoter methylation (Noushmehr et al. 2010). MGMT is a DNA methyltransferase, which conveys temozolomide (TMZ) resistance. Silencing of this gene via promoter methylation is a proposed marker predicting TMZ treatment response in gliomas (Hegi et al. 2005).

1.2.1.3 Anaplastic astrocytoma

Anaplastic astrocytomas (WHO grade III) can arise from diffuse astrocytomas (grade II) or arise *de novo* (without prior evidence of disease). They are generally found in adults as opposed to young adults as in the case of diffuse astrocytomas. The incidence is 0.35 per 100 000 population per year (Smoll and Hamilton 2014). As suggested the diffuse and anaplastic astrocytomas have many features in common. The histopathology is to a high extent similar with anaplastic astrocytomas being distinguished by their increase in cellularity, nuclear atypia and mitotic activity (Ki67/MIB-1 index 5-10%). There is higher variation in nuclear size, shape, coarsening, chromatin dispersion and increase in the prominence and number of nucleoli (Louis et al. 2007). The genetic alterations seen in anaplastic astrocytomas are largely similar to those found in diffuse astrocytomas, including *TP53*, *IDH1* and *ATRX* mutations accompanied by G-CIMP, but with the addition of loss of heterozygosity (LOH) and mutations affecting *PTEN* (Louis et al. 2007; Yan et al. 2009; Noushmehr et al. 2010; Kannan et al. 2012; Liu et al. 2012). Anaplastic astrocytomas subsequently develop into secondary glioblastomas usually within a 2-year period (Louis et al. 2007).

1.2.2 Oligodendrogliomas

1.2.2.1 Oligodendroglioma

Oligodendrogliomas (WHO grade II) are diffusely infiltrating well-differentiated tumors, typically located to the cerebral hemispheres most often affecting adults, representing 5-6% of gliomas. They have an incidence of 0.27-0.35 per 100 000 population per year. As the name suggests oligodendrogliomas are characterized by round cells with a uniform round nucleus and clear cytoplasm resembling oligodendrocytes accompanied by a moderate cellularity with a "honeycomb" appearance of the tumor area. Cell proliferation is low usually below 5% but there is typically a dense network of thin-walled branching capillaries and a presence of microcalcifications (Louis et al. 2007). Oligodendrogliomas can be confused with reactive and other neoplastic lesions but oligodendrogliomas carry their own characteristic genetic hallmarks. *IDH1* mutation is a required feature (Yan et al. 2009), just as in astrocytomas, however oligodendrogliomas do not normally carry mutations in *TP53* and *ATRX* (Cryan et al. 2014), but rather are defined by a co-deletion of chromosome arms 1p and 19q (1p/19q deletion) which results from a whole arm translocation event involving these same chromosomes. Oligodendrogliomas can progress to anaplastic oligodendrogliomas although this seems to occur somewhat slower than the progression from diffuse to anaplastic astrocytoma (Louis et al. 2007).

1.2.2.2 Anaplastic oligodendroglioma

Anaplastic oligodendrogliomas (WHO grade III) are similar to oligodendrogliomas, they occur slightly later in life (7-8 years) but share histological and genetic features with the latter. The annual incidence is around 0.07-0.18 per 100 000 population. Anaplastic oligodendrogliomas can develop from a lower malignant lesion or *de novo*. Tumors show a marked nuclear atypia and brisk mitotic activity, microvascular proliferation and a high cellularity accompanied by necrosis. Distinguished from glioblastoma by the occurrence of microcalcifications, a branching capillary network and the characteristic oligodendroglial tumor cells with a rounded hyperchromatic nucleus, perinuclear halo and few cellular processes (Louis et al. 2007). Anaplastic oligodendrogliomas are not believed to readily progress to glioblastomas, but it has been reported (Hiniker et al. 2012), and they have a better prognosis than anaplastic astrocytomas. Key genetic features include *IDH1* mutation and 1p/19q deletion as observed in oligodendrogliomas (Louis et al. 2007; Yan et al. 2009). In addition anaplastic oligodendrogliomas can show amplifications of *EGFR*, *PDGFRA*, *MYC*, *MYCN*, *CDK4*, *MDM2*, *MDM4* and deletions/mutations of *CDKN2A*/*B*, *CDKN2C*, *PTEN*, *RB1* which would explain the malignant progression, however these events are not as common as they are in glioblastomas (Louis et al. 2007).

1.2.3 Glioblastoma

Glioblastoma (WHO grade IV) is the most aggressive and frequent brain tumor in adults. Most commonly it is a primary tumor arising *de novo* with peak age of incidence between 45 and 75 years of age (mean age 61.3). The incidence is 3-4 per 100 000 population per year.

Glioblastomas are characterized by nuclear atypia, cellular pleomorphism, mitotic activity, vascular thrombosis, microvascular proliferation and necrosis. They account for 12-15% of all intracranial neoplasms and 60-75% of astrocytic tumors. They are most often localized in the subcortical white matter of the cerebral hemispheres with combined fronto-temporal lobe location being the most common. However glioblastoma cells migrate along white matter tracts, much as developing progenitor cells in the brain do, into regions such as the corpus callosum and along vasculature often resulting in the tumor engaging large regions of the brain expanding into multiple lobes and the contra lateral hemisphere. The clinical history is often as short as three months when the tumor arise *de novo*, referred to as primary glioblastoma. Glioblastomas can also develop from diffuse and anaplastic astrocytomas and are then referred to as secondary glioblastoma (<10% of all glioblastomas). While glioblastomas often share several histopathological features with diffuse and anaplastic astrocytomas, hence they are grouped together as astrocytic tumors (Louis et al. 2007). This is most likely a misleading view of glioblastomas, since they display a very wide array of additional histological and molecular features. Current research dictates that primary and secondary glioblastomas should be regarded as two different diseases (Parsons et al. 2008; Brennan et al. 2013). Prominent microvascular proliferation and/or necrosis are essential for the diagnose, this is often accompanied by heterogeneous features ranging from epithelial structures to poorly differentiated cells. Epithelial like structures are referred to as adenoid, containing cells with large oval nuclei, prominent nucleoli and round well-defined cytoplasm forming glandular structures. Several cellular compositions can be observed in glioblastomas e.g. with small elongated cells densely packed with hyperchromatic nucleoli, modest atypia, low level of GFAP but high proliferative activity (small cell glioblastoma). Like oligodendroglioma glioblastomas can contain cells reminiscent of oligodendroglial cells. Giant cell glioblastomas contain a predominant fraction of large multinucleated cells, which are also a common feature of glioblastomas but then not the dominating cell type. Gemistocytes found in diffuse and anaplastic astrocytomas are also often found in glioblastomas. Other cell features include granular cells and lipidized cells. Perhaps the most extreme histological features are those found in gliosarcomas with a mixture of gliomatous and sarcomatous tumor tissues sometimes even containing tumor cells with mesenchymal differentiation such as the formation of cartilage, bone and muscle (Louis et al. 2007).

For decades, glioblastoma patients have been managed with a combination of neurosurgery, standard-of-care chemotherapy/radiotherapy and with additional experimental therapies; however, the disease is incurable and patient survival remains dismal at a median of 12-15 months (Stupp et al. 2005; Van Meir et al. 2010). Enumerable studies have been published correlating histologic subclasses with clinical outcomes in attempts to provide meaningful prognostic indicators. Unfortunately, these studies were usually in conflict with each other as in the case of giant cell glioblastoma where some data suggested a worse prognosis while others highlighted a better clinical outcome attributed to a less infiltrative tumor growth pattern (Huang et al. 1996; Louis et al. 2007). Similarly, limited studies on gliosarcomas have hinted at a more favorable prognosis but later larger clinical trials failed to demonstrate such

a benefit (Meis et al. 1991; Galanis et al. 1998; Han et al. 2010). Presently, histological subtypes of glioblastoma are clinically treated the same and outcomes remain poor across all categories.

1.2.3.1 Genetic alterations in Glioblastoma

Glioblastomas, like other types of cancer, carry a diverse set of genetic alterations in oncogenes and tumor suppressor genes. Glioblastoma is associated with a characteristic set of genomic alterations including *EGFR* amplification, *EGFRvIII* deletion, chr7 gain, *CDKN2A*/*B* deletion, LOH of chr10, *PTEN* LOH/deletion/mutation, *CDK4* amplification, *PDGFRA* amplification/mutation, *MDM2* amplification, *MDM4* amplification, *MET* amplification, *CDK6* amplification, *PIK3CA*/*PIK3R1* mutations, *NF1* mutation/deletion, *RB1* mutation/deletion and *TP53* mutation/deletion. The end results of these genetic events are; 88-90% of cases with altered RTK/RAS/PI3K signaling leading to AKT and/or MAPK pathway activation, 86-87% of cases with altered p53 activation/signaling impairing normal p53 response, 78-79% of cases with altered RB signaling uncoupling normal cell cycle control and restriction (TCGA 2008; Brennan et al. 2013).

There is also a subset of *de novo* glioblastomas with *IDH1* mutations however it is likely that these tumors represent secondary glioblastoma despite the lack of a previous low-grade tumor detected in the patients (Parsons et al. 2008; Yan et al. 2009). These tumors, just like secondary glioblastomas, which also carry *IDH1* mutations, show a higher frequency of *TP53* and *ATRX* mutations as well as displaying the *IDH1* mutation induced glioma-CpG island methylator phenotype (G-CIMP) all of which are more related to astrocytomas than to primary glioblastoma (Noushmehr et al. 2010; Kannan et al. 2012; Liu et al. 2012; Brennan et al. 2013). As low grade gliomas progress there is an accumulation of genetic events found in glioblastomas; *RB1* mutation/loss, *CDKN2A* loss and *CDK4/6* amplifications seem to be related with the early stages of progression whereas *PTEN* loss, *PDGFRA* amplification and *EGFR* amplification are proposed to finally transform the lower grade lesion into a glioblastoma (Louis et al. 2007).

Glioblastomas display great inter and intra tumor heterogeneity also at the genetic level. Single tumors have been observed to contain different cell clones with unique genetic events such as *EGFR*, *PDGFRA* and *MET* amplifications. This heterogeneity is likely to pose problems for treatments, with different cell populations possibly relying on different cell signaling pathways (Snuderl et al. 2011). The evolution of different tumor cell clones could imply a functional relationship between them, possibly a dependence on each other, opening the door for developing treatments targeting tumor cell-to-tumor cell interactions.

Despite that glioblastomas have a well defined genomic landscape with *EGFR*, *PDGFRA*, *CDKN2A*, *PTEN*, *TP53*, *RB1* and *NF1* aberrations described relatively long ago new genetic events such as *IDH1*, *ATRX* mutations and now more recently the mutations of the *hTERT* promoter and loss of *QKI* are still being added (Louis et al. 2007; Parsons et al. 2008; Brennan et al. 2013). Continuous efforts and more knowledge are bound to further identify

genetic events important in gliomagenesis. This fact points to the need for molecular based subclassifications and further molecular based functional analysis of glioblastomas.

1.2.3.2 Molecular subclasses of glioblastoma

An updated approach to the molecular subclassification of glioblastoma was presented by the seminal works of TCGA Research Network, which among other things, highlighted four novel molecular subclasses based on gene expression in a large cohort of glioblastoma tissue samples (TCGA 2008; Verhaak et al. 2010; Brennan et al. 2013). These studies showed that molecular subclasses could be associated with different neural cell types and cancer pathways as well as differential response to more or less aggressive clinical therapies. Interestingly, similar glioma subclasses were first described as a progression model, although this study combined anaplastic astrocytomas (grade III) and glioblastomas (Phillips et al. 2006). This progression model has not been proven valid for the TCGA subclasses based on *de novo* glioblastomas.

TCGA showed by mRNA expression profiling that glioblastomas could be grouped into four molecular subclasses, namely: Classical, Proneural, Neural and Mesenchymal (not correlated with WHO histological subtypes). The subclasses are linked to different gene expression signatures with Neural being more close to normal brain and astrocytes, Classical and Proneural more represented by proliferation and neural progenitor markers. Proneural correlated more with oligodendrocytes than astrocytes whereas the opposite was true for Classical, Mesenchymal includes high expression of both mesenchymal and inflammatory cell markers and resembles cultured astroglia (Verhaak et al. 2010). Given that non-tumor cell gene signatures influence these subclasses, the signatures in part reflect the contaminating presence of normal neural and glial cells, necrosis, vascular proliferation, inflammatory cells and other non-tumor cell types. There are some genetic correlations to the mRNA expression subclasses, *IDH1* and *ATRX* mutated tumors fall within the Proneural subclass, *IDH1* mutations also go hand in hand with G-CIMP. Regarding other genetic alterations, such as *EGFR* and *PDGFRA* amplifications proposed to be linked to Classical versus Proneural subclasses and *RB1*/*NF1* alterations in the Mesenchymal subclass, the correlations are not always as strong (Fig. 2) (TCGA 2008; Verhaak et al. 2010; Brennan et al. 2013). When molecular subclasses were correlated with patient survival in response to more or less intensive therapeutic strategies, differences in survival were noted suggesting but not strongly supporting a biologic relevance to these subclasses. The Classical subclass had greatest benefit of aggressive treatment whereas there was none in the Proneural (Verhaak et al. 2010). In the TCGA follow-up study it is shown that G-CIMP/*IDH1* mutation are related to and predominantly explain the survival advantage noted in the Proneural subclass. Interesting in regard to the previous report concerning the benefit of aggressive treatment in the Classical subclass, *MGMT* promoter methylation was found to be a predictive biomarker in the Classical subclass but not in others (Brennan et al. 2013).

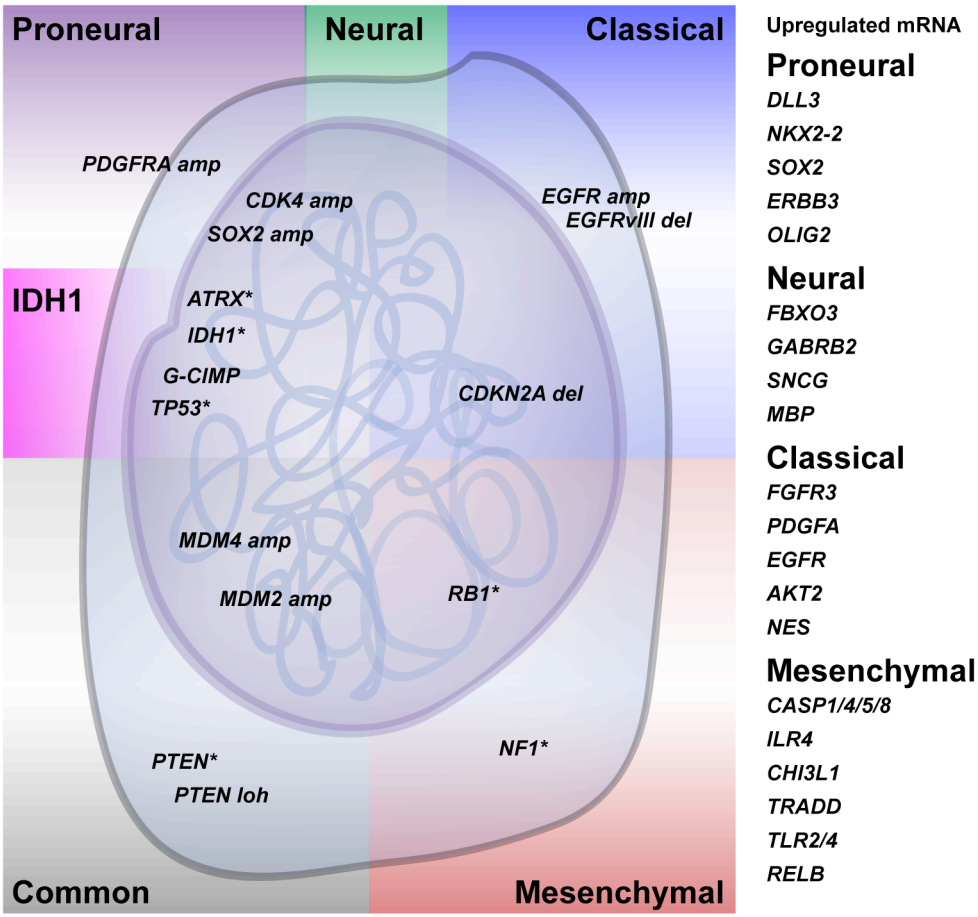

Figure 2. TCGA gene expression subclasses Proneural, Neural, Classical and Mesenchymal. IDH1 mutated tumors have here been given their separate class. Common includes frequently altered genes. Right panel includes genes with upregulated mRNA highlighted for each expression subclass *mutated, amp: amplified, del: deleted, loh: loss of heterozygosity.

As these studies were based entirely on tissue samples, some limitations include genomic contribution of non-tumor components such as inflammatory cells, blood vessels, microglia as well as non-neoplastic brain constituents like astrocytes, oligodendrocytes and neurons (TCGA 2008; Verhaak et al. 2010). The initial TCGA study defining the expression subclasses did reconfirm the presence of the Proneural, Classical and Mesenchymal subclasses in a small cohort of xenografts (Verhaak et al. 2010), however there could still be issues with non-tumor cell contaminations in such a setting. Importantly, given the intra tumor heterogeneity of glioblastoma it is unknown how well the region selected for analysis will represents the rest of the tumor (Snuderl et al. 2011; Patel et al. 2014).

Nonetheless the emergence of these mRNA expression based subclasses has helped to drive research in the field, especially in regards to correlation of glioblastoma genomics with mRNA expression patterns. The most recent attempt in the TCGA work has been to add a new large scale proteomic screen to evaluate activated pathways in the different glioblastoma subgroups (Brennan et al. 2013). MAPK pathway activation showed some increase in the Mesenchymal subclass whereas the PI3K pathway was more active in the Proneural and both groups had mTOR pathway activation. The initial results pointed to a very complex and non-

linear relation between expression subclasses, genetic profiles and protein levels, however the addition of protein analysis will surely help to establish more biologically relevant glioblastoma subgroups and identify drug targets in future studies.

1.2.3.3 Cancer stem cells in glioblastoma

Glioblastoma cancer stem cells (CSCs) are the population of cells with the potency to form neurospheres, when cultured under NSC conditions, showing multi lineage (neuronal-, astrocytic- and oligodendroglial-like) differentiation *in vitro*. To be considered true CSCs they should form tumors, recapitulating the founding tumor, when injected into the brains of immunodeficient NOD/SCID mice (Singh et al. 2003; Singh et al. 2004).

Even though the term CSCs has been accepted for variants of leukemia there is still a debate regarding CSCs in solid tumors. Stem cells, whether it comes to cancer or normal stem cells, are functionally defined as cells with the capacity of self-renewal and multipotency. There are two scenarios for the generation of these CSCs; The first being mutation of cells that already have reached their differentiated and mature state, causing dedifferentiation leading to the generation of cancer cells with stem cell attributes. A second possibility is the initial mutation taking place in stem cells generating cancer cells that already possess the attributes of self-renewal and multipotency (Singh et al. 2004). Stem cells continuously self-renew throughout our life span and would thereby be more likely to accumulate mutations. Even if the origin of CSCs is not defined, cancer cells with stem cell properties are clearly found in various types of cancers including glioblastoma (Lapidot et al. 1994; Al-Hajj et al. 2003; Singh et al. 2003; Singh et al. 2004).

An ongoing challenge with glioblastoma CSCs has been to identify a robust defining biomarker that can be used for prospective isolation and study of such cells. Initial studies of the surface marker PROM1 (CD133/AC133/Prominin-1) suggested that $PROM1^+$ cells were responsible for repopulating tumors while $PROM1^-$ cells did not have this capacity (Singh et al. 2003; Singh et al. 2004). In time, this however proved to be less reliable as subsequent studies showed that both $PROM1^+$ and $PROM1^-$ populations were able to generate tumors following transplantation into mice (Beier et al. 2007; Shmelkov et al. 2008). This has also been the case for other markers described in the literature. Despite the absence of a single defining biomarker, there is high expression of a large number of stem cell associated genes, e.g. NESTIN, GFAP, SOX2, A2B5, NANOG, OKT4, CD44 and KLF4 in glioblastomas (Ben-Porath et al. 2008; Ogden et al. 2008; Anido et al. 2010; Holmberg et al. 2011; Elsir et al. 2013). Several of these are transcription factors linked to embryonic development and stem/progenitor cells suggesting an activity of stem/progenitor cell associated transcriptional networks in glioblastoma. Genetically engineered mouse models (GEMMs) have also provided extra support to CSCs residing in the tumors giving rise to transient amplifying cells and differentiated progeny in a fashion similar to NSCs (Alcantara Llaguno et al. 2009). It remains unclear how locked the glioblastoma cells are to their specific stem cell state and other possible states along the differentiation pathway such as progenitor and terminally differentiated cell states. To add more complexity to the stem cell markers new research has

shown that many are context dependent acting together with other factors to exert their final function e.g. GFAP is expressed in stem cells as well as in differentiated astrocytes potentially serving different functions (Codega et al. 2014).

The CSC theory has been proposed as an alternative to the clonal evolution theory in cancer (Nowell 1976), where instead of there being the dominance of advantageous clones that prevail there is a small percentage of CSCs sustaining tumor growth. The two theories are also combined where CSCs and clonal evolution are both contributing biologic forces, which together with the tumor microenvironment and interclonal cooperativity give rise to these heterogeneous tumors (Hanahan and Weinberg 2011; Valent et al. 2012).

1.2.3.4 Glioblastoma cell of origin

The glioblastoma cell of origin has long been debated. Theories about and identification of CSCs in glioblastoma have certainly refueled this question. Reviewing the literature in regards to glioma models and especially GEMMs it is more likely that glioblastomas arise from transformed neural stem/progenitor cells than from differentiated neural cells. GEMMs utilizing stem/progenitor associated genes such as Gfap and Nestin for cell targeting illustrate that cells expressing these genes can be transformed into glioblastomas (Uhrbom et al. 2005; Hede et al. 2009; Chow et al. 2011). This transformation process more readily occurs in stem cell niches of the adult brain, such as the SVZ and can be induced by a number of glioblastoma associated genetic events (Swartling et al. 2012). Mouse glioma cells have also been shown to be dependent on Olig2 function similar to normal oligodendrocyte lineage cells and raising the possibility that glioblastoma arise from this lineage (Ligon et al. 2007; Mehta et al. 2011). In addition to niche progenitors the fact that $NG2^{+}$/$Olig2^{+}$ progenitor cells are the most abundant dividing cells in the brain suggest that these may be source of glioblastoma. Even though GEMMs are not as prone to form glioblastomas when differentiated cells are targeted under more physiological conditions, it is possible to transform neurons into glioblastoma cells (Friedmann-Morvinski et al. 2012).

1.2.3.5 DNA and histone modifications in glioblastoma

Epigenetics has had a popularity boom in cancer research during recent years. Epigenetics is a concept describing non-genetic changes resulting in gene regulation that is inherited by daughter cells. Basically what is referred to when using the word epigenetics is DNA and histone modifications such as methylation and acetylation. Stem, progenitor and differentiated cells will hold different epigenetic status resulting in specific gene regulation affecting cell identity and ultimately cell function (Hirabayashi and Gotoh 2010). Exactly how this works is not yet fully mapped and understood. In cancer there is a growing body of evidence that DNA/histone modifications and proteins involved in these processes are relevant to the disease (Jones and Baylin 2007; Nagarajan and Costello 2009).

DNA methylation occurs throughout the genome, it is believed that the methylation in and before promoter regions plays the largest role in gene regulation. Methylation takes place in CpG domains around gene promoters and enhancers (McClelland and Ivarie 1982). A

methylated promoter subsequently often results in a silenced gene and this has been shown for *p16*/*CDKN2*, *RB1* and *PTEN* in gliomas (Costello et al. 1996; Nakamura et al. 2001; Baeza et al. 2003). There is a growing interest in the DNA methylation patterns of cancer and it is becoming clear that the methylation patterns in the cancer genome are much more complex than first thought and not only confined to promoter regions (Eckhardt et al. 2006). The latest work from TCGA includes the methylation status of a number of glioblastomas and investigates possible correlations between methylation status and molecular subclass (Brennan et al. 2013). DNA methylation, gene expression subclass and genetic events are however not shown to correlate except when it comes to the *IDH1* mutated tumors showing G-CIMP which is also the case for low grade gliomas with *IDH1* mutation (Noushmehr et al. 2010; Brennan et al. 2013). *IDH1* mutation has been suggested to affect the general DNA methylation status of the cancer cell (Sasaki et al. 2012). Another example is loss of DNA (cytosine-5-)-metyltransferase (DNMT), an enzyme that normally establishes methylated cytosine residues. Loss of DNMT function leads to the hypomethylation of the cancer cell genome and deregulated gene expression together with increased genomic instability (Eden et al. 2003).

Histone modifications are fundamentally able to influence gene expression. The nucleosome, approximately 146 bp of DNA wrapped approximately twice around an octamer of the four core histone proteins (H2A, H2B, H3, H4) is the basic subunit of chromatin (Luger et al. 1997). Methylation and acetylation are the most described histone modifications (Turner 2005), this will together with certain proteins such as heterochromatin protein HP1 and linker histone H1 regulate if the chromatin is configured as euchromatin or heterochromatin and thereby controlling gene transcription (Nielsen et al. 2001). The most studied and perhaps the most important histone modifications are methylations of H3K9, with the addition of two methyl groups H3K9me2 being the first more reversible step of gene silencing and the subsequent addition of yet another methyl group, giving three in total, that is H3K9me3 representing fully silenced DNA and heterochromatin compaction (Tachibana et al. 2001). Histone methylation of H3K27 has also been linked to gene repression whereas methylation of H3K4, K36 and K79 has together with acetylation of histones been implicated in transcriptional activation (Clarke et al. 1999; Nakamura et al. 2002; Krogan et al. 2003; Krogan et al. 2003). There are histone modifiers deregulated and mutated in glioblastoma like histone deacetylases (HDACs) (Lucio-Eterovic et al. 2008; Parsons et al. 2008), there are mutations targeting the histones themselves e.g. H3.3 (Schwartzentruber et al. 2012), although found in pediatric gliomas not in adult glioblastomas (Brennan et al. 2013), and there are amplifications and deletions in genes regulating H3K9 methylation in medulloblastoma (Northcott et al. 2009).

1.2.3.6 Glioblastoma patient derived cell lines

The methodology for establishing PDCLs from patient tissue was first pioneered through CSC studies in glioblastoma. Cultures enriched for CSCs were created by culturing glioblastoma cells in NSC conditions (Singh et al. 2003; Galli et al. 2004; Singh et al. 2004).

Glioblastoma cells are grown in serum free media supplemented with recombinant human (rh) EGF and rh bFGF on non-adherent plates like NSCs. Glioblastoma cells can then as NSCs form neurospheres (or rather tumorspheres). This is in contrast to cultures utilizing serum containing media and adherent plates that induce differentiation in NSCs. Historical glioblastoma cell lines grown in serum, e.g. U87MG, grown in culture for many years lack genomic and mRNA expression profiles that are similar to patient samples and do not recapitulate the histopathology as xenografts (Sakariassen et al. 2006; Li et al. 2008; Hodgson et al. 2009). In contrast, glioblastoma cells grown under NSC conditions (PDCLs/CSCs) have been shown to faithfully recapitulate morphologic and immunophenotypic profiles of their tumors of origin both *in vitro* and *in vivo* (Galli et al. 2004; Lee et al. 2006; Wakimoto et al. 2011). However the extent and degree to which large number of cell lines may exhibit this fidelity has not been extensively tested.

Hence, stem cell conditions are proposed to maintain the stem/progenitor cell phenotype, whereas serum cultured lines are suggested to undergo a form of differentiation leading to the generation of lines with a more "clonal/restricted" cell phenotype (Singh et al. 2003; Singh et al. 2004; Lee et al. 2006; Pfenninger et al. 2007; Li et al. 2008; Hodgson et al. 2009). Glioblastoma cell lines cultured in NSC conditions are after a few number of passages devoid of non-tumor cell contamination. Thus, they represent the ideal model to identify true cancer cell intrinsic characteristics of glioblastoma.

1.3 MEDULLOBLASTOMA

Although not a glioma this is a grade IV brain tumor worth mentioning in the context of this thesis. Medulloblastomas are malignant invasive embryonal tumors of the cerebellum. They arise mainly in children but also occur in teenagers/young adults with an incidence of 0.5 per 100 000 children less than 15 years per year. There are several histological subgroups of medulloblastoma; Desmoplastic/Nodular medulloblastoma, Medulloblastoma with extensive nodularity, Anaplastic and Large cell medulloblastoma. Medulloblastomas have their own genetic characteristics separating them from astrocytomas, oligodendrogliomas and glioblastomas with *MYC* being one of the most common amplifications associated with large cell medulloblastoma and poor clinical outcome. Sonic hedgehog (SHH) signaling is also strongly associated with medulloblastomas of the desmoplastic variant most commonly exhibiting loss of *PTCHD* which is an inhibitor of the SHH pathway. There are also similarities between medulloblastomas and gliomas such as; *CDK6* gain and deletions of *PTEN* and *CDKN2A* (Louis et al. 2007). There are four molecular subclasses of medulloblastoma; WNT, SHH, Group 3 and Group 4 associated with activation of different pathways and patient demographics (Cho et al. 2010; Northcott et al. 2010). Medulloblastomas of the WNT subclass arise outside the cerebellum from cells of the dorsal brain stem whereas SHH subtype tumors arise from within the cerebellum (Gibson et al. 2010). SHH tumors have been proposed to originate from the granular neuron precursor cells (GNPCs) (Schuller et al. 2008). PROM1 expression and its association to CSCs is another

feature shared between medulloblastomas and glioblastomas (Singh et al. 2003; Singh et al. 2004; Raso et al. 2011).

1.4 PROMININ-1 (PROM1/CD133)

Prominin-1 was the first 5-transmembrane (5-TM) protein identified in the prominin family (Miraglia et al. 1997; Mizrak et al. 2008). Since its discovery Prominin-1 has been described by several research groups as a stem cell marker although the amount of evidence for this in the normal nervous system has been very limited and surpassed by the interest in the cancer stem cell community (Bauer et al. 2008). PROM1 is a single-chain polypeptide of 865 amino acids with 5-TM regions, extracellular N-terminus and cytoplasmic C-terminus and two extracellular loops with eight sites each for N-linked glycosylation (Miraglia et al. 1997). PROM1 was first discovered in human hematopoietic progenitor cells but later also described in mouse tissue (Miraglia et al. 1997; Weigmann et al. 1997). Mouse Prom1 has only a 60% amino acid identity with the human PROM1 but on the other hand has a very similar protein structure; 5-TM, 858 amino acids, extracellular N-terminus, cytoplasmic C-terminus, two extracellular loops with eight N-glycosylation sites (Weigmann et al. 1997). Prominin-1 has been detected in many different tissue types; brain, intestine, kidney, bone marrow, heart, liver, lung, pancreas, placenta, skeletal muscle, and testis either through mRNA or antibody (Corbeil et al. 2001). At least two splice variants exist for human PROM1 and as many as 8 splice forms have been described for mouse Prom1 (Fargeas et al. 2004). Prominin-1 can often be seen in association with plasma membrane protrusions such as microvilli, in rod photoreceptor cells invaginations and myelin sheaths (Maw et al. 2000; Yang et al. 2008; Corbeil et al. 2009). Evidence suggests that Prominin-1 interacts with microdomains known as lipid rafts in the plasma membrane (Corbeil et al. 2001). Mutations in *PROM1* are believed to cause retinal degeneration (Maw et al. 2000).

PROM1 has been associated with CSCs in a wide number of cancers but particularly in the CNS (Lapidot et al. 1994; Al-Hajj et al. 2003; Fargeas et al. 2004; Singh et al. 2004; Ding et al. 2013). Even though researchers have shown great interest in studying Prominin-1 there is limited knowledge about the proteins actual function and its expression across the differentiation spectrum of cells.

1.4.1 Prominin-1 in embryonic CNS development

Prominin-1 expression was initially reported in the VZ, retina and spinal cord of the developing CNS (Corbeil et al. 2000; Uchida et al. 2000; Corbeil et al. 2009) although the evidence for its expression in normal and well defined stem cells *in vivo* has been limited by difficulty in detecting the protein. $PROM1^+$ cells isolated from the human fetal ventricular zone have the ability to generate neurospheres, which retain self-renewal and multi-lineage differentiation capacity (Uchida et al. 2000). During mouse embryonic brain development splice variant 1 (s1) is the dominant form of *Prom1* (Corbeil et al. 2009). Previous reports suggested that $Prom1^+$ cells isolated from the perinatal (P7) mouse cerebellum were multipotent neural stem cells (Lee et al. 2005). However each of these methods did not

specifically isolate the Prom1$^+$ cells and were likely containing other progenitor cells from the SVZ/other regions, which readily generate neurospheres, calling into question the actual potential of Prom1$^+$ cells *in vivo*.

1.4.2 Prominin-1 in the adult CNS

In the adult brain, Prominin-1 expression has been reported in ependymal cells and murine hippocampus (Coskun et al. 2008; Walker et al. 2013). In transgenic Prom1-LacZ mice, Prom1/LacZ was co-expressed with Gfap in cells of the SVZ having properties of multipotent self-renewing neural stem cells (Coskun et al. 2008). That Prom1$^+$ cells are stem/progenitor cells from the SVZ/VZ is supported by other recent studies (Codega et al. 2014; Khatri et al. 2014). However, Prom1/LacZ$^+$/Gfap$^-$ cells single-sorted from this region were not able to form secondary neurospheres or to differentiate into all neural lineages (Coskun et al. 2008). Prom1/LacZ expression was also noted in cells with non-stem cell phenotypes widely throughout the adult mouse brain regions but whether the endogenous gene is expressed in a similar pattern was not fully established and artifacts of transgenic expression make determination of this difficult (Beckervordersandforth et al. 2010; Ding et al. 2013). In regards to examination of Prom1 expression in normal brain it has been identified in myelin sheaths and *Prom1* s3 mRNA was identified in cells expressing Olig2 (Corbeil et al. 2009). Studies in different organ systems (e.g. retina, prostate and hematopoietic systems) have also highlighted the non-stem cell expression and function of Prominin-1 (Missol-Kolka et al. 2011; Arndt et al. 2013; Gurudev et al. 2013).

It is suggested that significant differences may exist between mouse and human Prominin-1 (Wang et al. 2013). There are also discrepancies between the PROM1 mRNA and protein expression reported in human colon tissue due to differences in glycosylation affecting antibody specificity (Kemper et al. 2010). These are issues that should be taken into account, especially since diverse splice variants and differences in glycosylation have been reported to distinguish different cell types/cell states and are likely to reflect fundamentally diverse functions of Prominin-1 (Corbeil et al. 2009; Kemper et al. 2010).

1.4.3 Prominin-1 in glioblastoma

Prominin-1 is believed to identify tumor-initiating cancer stem cells in a wide range of cancer types including leukemia (Lapidot et al. 1994), breast (Al-Hajj et al. 2003) and glioblastoma (Singh et al. 2003). The cancer stem cell hypothesis suggests that only a minor subpopulation of the tumor cells maintain tumor growth and have the indefinite capacity to self-renew. PROM1 is ubiquitously referred to as such a marker expressed in only a minor subpopulation of stem cells in glioblastoma. Based on flow cytometry analysis, PROM1$^+$ cells in glioblastoma have been described as tumor initiating cells able to propagate tumor growth in immunodeficient NOD/SCID mice xenograft models and confer radioresistance (Singh et al. 2003; Singh et al. 2004; Bao et al. 2006; Kang and Kang 2007). However, glioblastoma PROM1$^-$ cells can also contribute to tumor propagation (Galli et al. 2004; Beier et al. 2007; Joo et al. 2008; Wang et al. 2008). This raises the possibility that Prominin-1 may not be as

closely linked with "stemness" or tumor initiating phenotype in normal cells or cancer cells as previously proposed. Recent studies of Prominin-1 have used alternatives to flow cytometry, which allow more direct *in situ* visualization of its expression. Such studies increasingly describe differences in expression of the multiple and complex Prominin-1 isoforms in mouse and human. These studies have also increasingly highlighted Prominin-1 non-stem cell functions in the hematopoietic, retinal and prostate systems (Missol-Kolka et al. 2011; Arndt et al. 2013; Gurudev et al. 2013). In addition, expression of Prominin-1 has been reported as regulated by hypoxia, supporting the possibility that Prominin-1 may be a dynamically regulated protein not necessarily associated with cell lineage or stem cell phenotypes (Griguer et al. 2008).

Multiple studies have shown neurosphere formation or PROM1 antigen expression to be associated with shorter survival in patients and in mice transplanted with such tumor cells (Beier et al. 2007; Zeppernick et al. 2008; Laks et al. 2009). This would imply that PROM1 could serve as a prognostic marker however the biology behind this has not been explained.

1.5 NPM1 (NUCLEOPHOSMIN/B23)

Cancer cells, including glioma cells, display increasing nucleolar prominence and number alongside coarsening and dispersion of chromatin (Louis et al. 2007). Given this the nucleolus and nucleolar proteins hold a potentially interesting role in cancer. NPM1 is a non-ribosomal nucleolar protein that has been related to cancer (Grisendi et al. 2006). *Npm1* deficient mice display a defective haematopoiesis, and myelodysplastic syndrome (MDS), which is embryonic lethal (Grisendi et al. 2005; Grisendi et al. 2006). In addition, *Npm1*$^{-/-}$ embryos (E10.5) lack proper forebrain with the subdivision between metencephalon and mesencephalon shifted anteriorly. Analysis of neural tissues revealed marked apoptosis suggesting NPM1 serves a crucial function in normal brain development (Grisendi et al. 2005).

1.5.1 The nucleolus and nucleolar stress

Nucleoli are dynamic nuclear compartments rich in protein and RNA where the cell's ribosome biogenesis takes place (Hernandez-Verdun 2006). The nucleolus is said to mirror a series of metabolic changes in cancer cells and human tumors with nucleolar hypertrophy have worse prognosis (Derenzini et al. 2009). Of interest in relation to the recent finding regarding hTERT promoter mutation in glioblastoma (Brennan et al. 2013), is the fact that the nucleolus is the assembly place for the telomerase complex (Yang et al. 2002). Furthermore, *TP53* mutations and *RB* loss, also common in glioblastoma, are related a higher degree of nucleolar hypertrophy (Derenzini et al. 2004; Trere et al. 2004).

The nucleolus lacks a lipid bi-layer, and nucleoli form around ribosomal DNA (rDNA). The human rDNA genes are arranged in the form of tandem repeats at the chromosomal nucleolar organizer regions (NORs), there are around 400 copies of the rDNA gene in humans situated on the p-arms of the five acrocentric chromosomes 13, 14, 15, 21 and 22 (Boisvert et al. 2007; Shaw and Brown 2011). RNA polymerase I (RNA pol I) initiates transcription and this

leads to the formation of the nucleolus. The nucleolus is divided into three main subcompartments; fibrillar center (FC), dense fibrillar component (DFC) and granular component (GC). rDNA transcription is thought to occur at the border between the FC and DFC, with RNA pol I subunits predominately in the FC (Boisvert et al. 2007). RNA pol I transcribes 47S precursor RNA that is subsequently processed and cleaved into the 28S, 18S and 5.8S ribosomal RNAs (rRNAs). Small nucleolar ribonuclear proteins (snoRNPs) help process the rRNA subunits in the DFC region, which are then assembled in ribosome subunits in the GC of the nucleolus. This process involves a number of ribosomal proteins and 5S rRNA (Fig. 3) (Boisvert et al. 2007).

Nucleolar proteins are in constant flux between the nucleolus, nucleoplasm and cytoplasm (Lam et al. 2007). This flux of proteins has been assigned an important role in cellular stress signalling. The nucleolus responds to cellular stress that results in disturbances/defects in ribosome biogenesis (Olson 2004; Mayer and Grummt 2005; Boulon et al. 2010). This may be induced by mutated ribosomal/nucleolar proteins, a variety of chemotherapeutic drugs, irradiation, viral infection and heat shock (Draptchinskaia et al. 1999; Rubbi and Milner 2003; Burger et al. 2010). This is referred to as "nucleolar stress" or "ribosomal stress" (Fig. 3) (Zhang and Lu 2009). Nucleolar stress can lead to p53 activation resulting in cell cycle arrest, apoptosis, differentiation or senescence (Lindstrom and Zhang 2008; Drygin et al. 2009; Drygin et al. 2010; Morgado-Palacin et al. 2012). However there are p53 independent mechanisms also resulting in cell cycle arrest. Following nucleolar stress RPL11 is released from the nucleolus, RPL11 can destabilize E2F-1 and PIM1 kinase as well as inhibiting the expression of c-Myc and 5S RNA (Dai et al. 2007; Dai et al. 2010; Iadevaia et al. 2010; Donati et al. 2011). There are still plenty of additional proteins possibly associated with the nucleolus that have not yet been characterized but could play additional roles in nucleolar stress (Wool 1996; Lindstrom 2009; Warner and McIntosh 2009).

The nucleolus has also been assigned a role in organizing chromosome domains in the nucleus. Essentially, the nucleolus could be involved in the epigenetic and genetic regulation of the genome (McKeown and Shaw 2009; Bartova et al. 2010; Nemeth and Langst 2011).

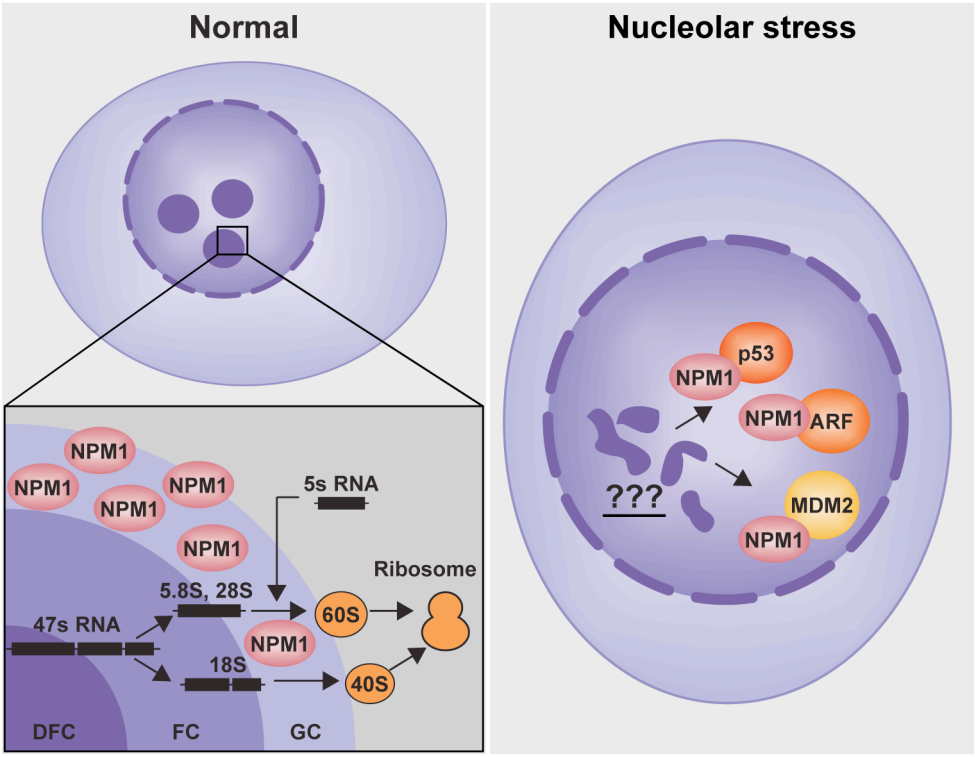

Figure 3. The nucleolus in the normal cell (left). rDNA transcription, rRNA processing and ribosome subunit assembly. NPM1 is located mainly in the GC acting as a chaperone. During nucleolar stress (right) NPM1 becomes dispersed from the nucleolus into the nucleoplasm where it inhibits MDM2 and stabilizes p53 and ARF. FC: fibrillar component, DFC: dense fibrillar component GC: granular component.

1.5.2 NPM1, a stress sensing nucleolar chaperone

NPM1 is a very abundant 37kDa phosphoprotein mainly localized in the nucleolus, more concentrated to the GC (Fig. 3). It takes part in the ribosome biogenesis and can shuttle between the nucleus and cytoplasm transporting pre-ribosomal particles (Grisendi et al. 2006). NPM1 may act as a histone chaperone given that it binds histones and assembles nucleosomes *in vitro* (Okuwaki et al. 2001; Namboodiri et al. 2004). However, the role of NPM1 in chromatin dynamics and ribosome biogenesis is not fully understood and NPM1 is not essential for rDNA transcription (Colombo et al. 2005; Maggi et al. 2008). NPM1 interacts directly with many cellular proteins and is involved in various cellular processes including centrosome duplication and mRNA splicing (Grisendi et al. 2006; Lindstrom and Zhang 2006; Okuwaki 2008). NPM1 staining can be used as a control for detecting nucleolar stress due to its strong association with the nucleolar rim, upon nucleolar stress induction the nucleolus becomes deformed in a way visible with NPM1 staining. After nucleolar stress induction ribosomal proteins (RPs) dissociate from the nucleolus and stabilize p53, this is also true for NPM1. NPM1 can directly stabilize p53, bind MDM2 inhibiting its degradation of p53 as well as stabilizing ARF leading to increased p53 activation (Fig. 3). It has been suggested that NPM1 is essential for the full p53 response (Colombo et al. 2002; Kurki et al. 2004). However, NPM1 is not the only nucleolar protein capable of mediating p53

stabilization following nucleolar stress (Lindstrom et al. 2007; Macias et al. 2010; Zhou et al. 2012).

Furthermore, NPM1 interacts with proteins involved in maintaining nucleolar structure. One such protein is CTCF, a sequence-specific DNA binding protein that delimits juxtaposed domains of active and inactive chromatin (Yusufzai et al. 2004; Zlatanova and Caiafa 2009). Its loss results in nucleolar fragmentation and reduced silencing of rDNA (Guerrero and Maggert 2011; Hernandez-Hernandez et al. 2012). NPM1 plays another role in maintaining DNA integrity during mitosis where it is involved in centrosome duplication. *Npm1*$^{-/-}$ mouse embryonic fibroblasts (MEFs) show centrosome amplification and increased genomic instabillity (Grisendi et al. 2005).

1.5.3 NPM1s dual role in cancer

NPM1 is frequently mutated in lymphoma and leukemia and found to be overexpressed in solid tumors, this suggests a dual role for NPM1 in cancer. High levels of NPM1 may help to conserve the structural integrity of nucleolar chromatin, functional rDNA transcription and ribosome biogenesis, whereas cells with reduced levels of nucleolar NPM1 or mutant NPM1 may suffer disruption of nucleolar chromatin organization, blunting of the p53 response and genomic instability (Grisendi et al. 2006).

1.5.3.1 Loss of NPM1 function in cancer

ARF is a nucleolar protein that binds and antagonizes MDM2 ubiquitin ligase activity for p53 (Pomerantz et al. 1998; Zhang et al. 1998). In turn, NPM1 co-localizes with and binds ARF protecting it from degradation (Korgaonkar et al. 2005). Thus, in the absence of NPM1, ARF is unstable and is less effective in activating p53 (Colombo et al. 2005; Colombo et al. 2006).

In AML, which often exhibits a certain NPM1 mutation (NPM1c^{+}) resulting in NPM1 localization to the cytoplasm. This would cause a scenario of decreased amounts of NPM1 in the nucleolus and lead to the NPM1c^{+} mediated translocation of ARF from the cell nucleus to the cytoplasm thereby preventing its p53 stabilization. AML associated NPM1 mutations often overlap with mutations in *DNMT3A* (TCGA 2013). Loss of function in DNMTs (DNMT1/DNMT3B) have been associated with elevated levels of rRNA primary transcript (Majumder et al. 2006).

Depletion of NPM1 in *Arf*$^{-/-}$ MEFs and HeLa cells can also increase rDNA transcription (Apicelli et al. 2008; Maggi et al. 2008; Tafforeau et al. 2013). *Npm1*$^{-/-}$ cells are suggested more predisposed to transformation by oncogenes, such as Myc and Ras. NPM1 together with the F-box protein Fbw7γ is involved in the ubiquitination and proteasome degradation of c-Myc. Hence, NPM1 loss can stabilize c-Myc and result in enhanced cell growth (Bonetti et al. 2008).

1.5.3.2 NPM1 upregulation in cancer

NPM1 is highly expressed in a number of cancers but the functional consequences remain unclear (Colombo et al. 2011). In glioma there is evidence of NPM1 upregulation and it is reported that loss of NPM1 sensitized U87MG and A172 glioma cells to TMZ resulting in cell death and reduced colony formation (Gimenez et al. 2012). NPM1 was found to be critical for rDNA transcription in HeLa cells (Murano et al. 2008).

As discussed earlier gliomas have frequent alterations of pathways concerning p53 and ARF rendering the pathways inactive therefore gliomas could instead benefit from the growth promoting and chaperone functions of NPM1. Possible benefits of high NPM1 levels could be increased resistance to nucleolar stress, increased DNA stability and stable ribosome biogenesis. Hence, the ability of NPM1 to suppress apoptosis may promote cancer cell survival during tumor development (Grisendi et al. 2006). Furthermore, the possible role of NPM1 in histone and DNA modifications makes it interesting to investigate with regards to the suggested epigenetic states of gliomas (Brennan et al. 2013).

2 AIMS

The aims of the thesis were:

I. To generate a library of glioblastoma patient derived cell lines (PDCLs) grown under neural stem cell conditions with accompanying patient derived xenografts (PDXs) and evaluate these models in contrast to patient tumors.

II. Investigate Prominin-1 expression in the developing/adult CNS and in glioblastomas using RNA based in situ hybridization and evaluate antibody recognition of Prominin-1 expressing cells.

III. Investigate the nucleolar changes induced following NPM1 depletion in relation to morphology, chromatin structure and rDNA transcription.

IV. Investigate the possible roles of NPM1 in gliomas.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Integrative pathogenomic analysis of a glioblastoma cell line library reveals novel tumor cell intrinsic subclass distinctions

3.1.1 Results

We cultured 91 glioblastoma samples, 43% of samples processed (39 of 91) generated PDCLs with long term growth potential (greater than 5 passages) in non-adherent serum free EGF, bFGF supplemented NSC culturing conditions. A subset of these lines were further characterized, 22 PDCLs generated from *de novo* and 5 from recurrent glioblastomas, termed BT-lines. BT-lines and their corresponding patient derived xenograft (PDX) recapitulated patient tumors, shown by genomic and proteomic analyses. Common glioblastoma genetic alteration signatures were preserved including *EGFR*, *PDGFRA*, *MDM4*, *CDK4* and *CDK6* amplifications, *EGFRvIII*, *PTEN* and *CDKN2A* deletions and we also noted *QKI* (tumor suppressor) loss and *MYC* amplifications. BT-lines expressed glial stem/progenitor cell and glioblastoma associated proteins NESTIN, SOX2, OLIG2, GFAP confirming their glial nature.

BT-line PDXs did not only grow in an infiltrative pattern similar to that of patient glioblastomas but also matched several WHO described histological features of glioblastoma; classic, gemistocytic, giant cell, myxoid, oligodendroglial and poorly differentiated. This could also be linked back to founding tumors.

Expression profiling of BT-lines revealed 4 clinically relevant *in vitro* subclasses; A, B, C and D. Class A was associated with Wnt/B-catenin, TGF-B and BMP signaling with expression of genes; ACVR1, DKK3, FGFR1, HDAC9, SMAD7, SFRP1 and CAV1. Class B displayed a signature characterized by interferon signaling with high expression of; IFIT3, IRF9, DOK5, MUC1, IFITM1, OAS1 and TNFSF10. Notch signaling associated genes were detected in Class C; DTX1, HEY1, AKT3 and BCL7A. Class D expressed genes involved in p53 signaling and cell cycle checkpoints; BIRC5, MAD2L1, MTCH2, PLK4, CHEK2, MCM10, MYBL2 and NASP. Subclass comparisons revealed that *in vitro* BT-line classes resembled the TCGA Mesenchymal and Proneural *in vivo* subclasses, however they were not a four-group division of theses two subclasses. Class A most resembled the Mesenchymal subclass and class D the Proneural but none of the BT-line classes had strong correlations with the Classical and Neural subclasses. Expression profiles of individual BT-lines often associated them to several of the TCGA subclasses. BT-lines from recurrent glioblastomas destabilized clustering and hence were treated as their own class (class R).

We investigated correlations between BT-line expression classes, histological features, protein levels and genomics. Class A was the one class with no PDXs exhibiting necrosis, microvascular proliferation (MVP) or hemorrhage. Class B lacked *CDKN2A* deletions, which might be subsidized with *RB1* loss and subsequent mRNA down regulation (-2.9 in relation

to the rest of the BT classes) seen in this subclass. In Class B SOX2 was the most dominant IHC marker and PDXs had infiltrative growth patterns. BT-lines displaying a combination of giant cell and poorly differentiated histology did not have *CDKN2A* deletions despite belonging to different expression classes.

EGFRvIII deletions were found in class C and D, the *EGFRvIII* deletion was accompanied by a classic glioblastoma histology in 4/4 cases and round cell / oligo histology in 3/4 cases, *EGFR* amplification was seen in all classes except B. The one BT-line with *PDGFRA* amplification fell within class D, which was the one closest resembling the Proneural subtype. Class C gave rise to highly infiltrative xenografts while class D and R showed the highest presence of fast growing more stationary tumors possibly due to deranged regulation of cell cycle genes for class D and the selective pressure of previous treatment in class R.

SOX2 which has been suggested as a driver in glioblastoma was present in all BT-lines, there was however differences in its relation to other proteins. High SOX2 and OLIG2 seemed to be associated with class C (Notch signaling) while high SOX2, GFAP and NESTIN was seen in class D (p53 signaling and cell cycle). The same pattern was seen within class R with SOX2 /OLIG2 high in some BT-lines and SOX2/GFAP in others. Class R showed the highest levels of pAKT. Both $pAKT^{Thr308}$ and $pAKT^{Ser473}$ was significantly higher in BT-lines generated from recurrent compared to BT-lines generated from *de novo* glioblastomas.

We applied the top genes defining *in vitro* subclasses from *de novo* glioblastomas to TCGA patient tissue sample data, which generated 5 subclasses with differences in survival. Class 3 defined a group of patients that were older and had shorter survival. This was independent of *IDH1* mutational status and if the tumor was recurrent or not. Although the decrease in survival can be explained by the higher median age, class 3 identifies a biologically relevant group of older patients. Class 3 closest resembled BT-line class B and top upregulated genes in class 3 are involved in mitochondrial respiration, oligodendrocyte density, the BRCA1 network and genes often down regulated in Alzheimer's disease.

3.1.2 Discussion

This investigation of glioblastoma cell dynamics *in vitro* and *in vivo* demonstrates PDCLs and accompanying PDXs as a truly heterogeneous glioblastoma model concerning tumor cell intrinsic features and highly relevant for functional studies and drug screens.

Glioblastoma PDCLs cultured in NSC conditions and their PDXs are reported to represent the patient disease to a higher extent than PDCLs grown long term in serum culturing conditions (Bigner et al. 1990; Singh et al. 2004; Gunther et al. 2008). Our BT-line library proves this concept and further strengthens the notion that PDCLs to a very high extent, recapitulate the original disease. BT-lines glial origin could be confirmed using IHC leaving no doubt that these PDCLs were glioblastoma derived. PDXs grew in infiltrative manner and recapitulated several WHO described histological subtypes and features characteristic for glioblastoma pathology. The feature that was most under-represented in PDXs was MVP, which normally is one of the criteria in the diagnosis of glioblastoma. This needs

consideration if one sets out to investigate tumor-vasculature interactions in a PDX model. We can only speculate if the lack of MVP is a consequence of the interspecies, immunodeficient or spatial setting in PDX models.

The genomic landscape in BT-lines matched that described for glioblastomas (Brennan et al. 2013). The lack of amplifications conserved in serum cultured lines has been one of the main issues with those models (Bigner et al. 1990). *EGFR* amplifications were found in 38% of BT-lines and 44% of those carried the *EGFRvIII* deletion. This, including the retention of *PDGFRA*, *MDM4*, *CDK4* and *CDK6* amplifications will hopefully provide models applicable for developing therapies targeting these alterations. Genetic events rare in glioblastomas were seen to an even lesser extent in BT-lines, this would most likely be due to the limited number of BT-lines included in our study. Nonetheless, there could be an *in vitro* selection. There did appear to be enrichment for *CDKN2A* deletions, although this was not statistically significant. *CDKN2A* deletion has been associated with the enhanced ability to grow in cell culture (Hartmann et al. 1999). However, it was not a feature required for BT-lines as seen in class B with normal *CDKN2A* karyotype, although mutational status was not assessed.

Glioblastomas have been reported to consist of different cell populations with different amplified genes, such as *EGFR*, *PDGFR* and *MET* (Snuderl et al. 2011). Unfortunately we have not yet investigated this in BT-lines, however it would be of interest to know if this also could be sustained in PDCLs. Tumor heterogeneity and inter clonal cooperation have been proposed to promote disease progression. Heterogeneity is also described in regards to mRNA expression classes. Single cell sequencing showed that glioblastomas could harbor tumor cell populations representing all of the TCGA described expression subclasses (Patel et al. 2014). This raises the question of how valid TCGA expression subclasses are and suggests that differences seen in subclasses would just be dependent on what tumor region that was analyzed. The heterogeneity is also an issue in BT-lines, however due to reports that PDCLs grown in NSC conditions become devoid of non-tumor cells after 3-5 passages this should eliminate non-tumor cell contribution to expression profiles. With regards to this we were hoping that expression profiling of the BT-line library would reveal tumor cell intrinsic pathways activated in gliomas with greater biological value than those based on tissue samples.

We ended up with four BT-line subclasses after removing recurrent samples from the analysis. Recurrent samples destabilized the clustering so due to the logical conclusion that these samples might represent a different biology they were removed. The accuracy of doing so can be argued since if they would represent a similar biological divergence due to being from recurrent samples they should have clustered into their own class giving us 5 distinct subclasses. Either way, using PDCLs from *de novo* glioblastomas generated four subclasses. It was not so surprising to see that two of the BT-line subclasses (C and D) were associated with the Proneural subclass given the SOX2, OLIG2 and NSC associations of PDCLs. It was more unexpected to see that the Mesenchymal subclass correlated most with the other two (A and B) since it has been suggested that the Mesenchymal TCGA subclass could be associated

with inflammatory cells (Verhaak et al. 2010). We also saw high levels of TGF-B associated genes in BT-line class A suggesting that there is in fact a tumor cell intrinsic activation of this pathway and it is not a byproduct of inflammatory cells. Another surprise was the lack of a strong resemblance to the Classical subclass despite the retention of *EGFR* amplifications in BT-lines. This could be due to the addition of EGF in our cell cultures, canceling out differences in levels of EGFR signaling between different BT-lines. It could also be so that this is not the defining factor of that subclass since *EGFR* amplification is also found in other TCGA subclasses. BT-lines do give us a unique set of expression subclasses, both with similarities and distinctions from TCGA subclasses.

Interestingly, class R showed the highest levels of pAKT. Both $pAKT^{Thr308}$ and $pAKT^{Ser473}$ was significantly higher in BT-lines generated from recurrent compared to BT-lines generated from *de novo* glioblastomas. Implying increased AKT signaling, which has been linked to faster tumor recurrence and more aggressive tumors (Johnson et al. 2014; Robinson et al. 2014). This would suggest that mechanisms causing this increased AKT signaling are preserved in PDCL models. Hence, collections of BT-lines derived from recurrent tumors represent a system for addressing the recurrent disease specifically.

In order to investigate if our BT-line classes could add any insight to or stratification of clinical samples BT-line class defining genes where applied to the TCGA patient dataset. This led to further stratification of TCGA clinical samples and the isolation of subclass 3 defining a set of patients that were older and had a shorter survival not identified using the TCGA subclassification. Using *in vitro* PDCLs from glioblastomas to determine tumor cell intrinsic properties would then better describe the patient diseases than bulk tissue samples from the tumor.

Our cohort of PDCLs shows a great diversity with many key aspects of glioblastoma present. The main issue with further research using this model system will be to determine how many PDCLs of a given sort are needed for relevant studies i.e. how many PDCLs with the EGFRvIII variant are needed in order to evaluate a drug acting on said target. This will be dependent on what one decides to study, certain processes in the cell will be more or less redundant and my personal guess is that often one will need to target multiple pathways simultaneously. This will increase the number of PDCLs needed in order to target different constellations of activated pathways. It also remains to be determined if PDCLs will need to be investigated *in vivo* as PDX models in order for them to produce relevant results. Despite not recapitulating the true human physiology PDXs may add that extra degree of metabolic and microenvironment impact needed to better evaluate new drug treatments. There is also the issue of some PDCLs only being expandable *in vivo*. PDX models will be more expensive but regarding our limited understanding of microenvironment, metabolism, drug target availability and cancer cell interactions this might help us to better mimic the disease until we can grasp all concepts needed to fully construct relevant *in vitro* models.

3.2 PAPER II

Prominin-1 (CD133) defines both stem and non-stem cell populations in CNS development and gliomas

3.2.1 Results

In order to get more clarity in the expression patterns of Prominin-1 in embryonic/adult CNS and glioblastoma we utilized RNA in situ hybridization as complement to existing Prominin-1 targeting antibodies.

Using RNA *in situ* hybridization tools capable of detecting multiple isoforms of Prom1, we find evidence for two distinct *Prom1* cell populations in mouse brain. *Prom1* RNA is expressed in stem/progenitor cells of the ventricular zone and the spinal cord in embryonic brain. In adult mouse brain *Prom1* RNA is high in a rare widely distributed cell population (*Prom1*^{hi}) and low in SVZ/SGZ stem cell zones. *Prom1*^{hi} cells are found in both white and gray matter but there is a higher occurrence in white matter, reconfirmed by WB and qRT-PCR. Lineage marker analysis reveals that *Prom1*^{hi} cells predominantly co-label with Olig2 (98%) there is however a subset of *Prom1*^{hi} cells expressing Sox2 (10%). *Olig1/2* knockout mice retain *Prom1*^{hi} cells so unlike other Olig2 expressing cells *Prom1*^{hi} are not dependent on Olig gene function. Bromodeoxyuridine (BrdU) labeling identifies *Prom1*^{hi} as a slow-dividing/slowly generated cell population in the adult brain. The *Prom1*^{hi} population shows no increase in BrdU labeling after mechanical lesioning of the brain, neither does this cell population decrease or increase after irradiation.

PROM1 could also be identified in the human embryonic VZ and in cultured NSCs from the embryonic ganglionic eminence. In adult human brain, PROM1 cells are rarely positive for OLIG2, but express astroglial markers GFAP and SOX2. There is a large variability of PROM1 protein levels in our human glioblastoma PDCLs/PDXs library, from no expression to strong uniform expression. PROM1 is associated with GFAP, SOX2 and OLIG2 however association patterns vary across PDCLs. PROM1 populations can both be predominantly senescing or actively dividing as illustrated by Ki67 labeling. TCGA and PDX data show that high expression of *PROM1* correlates with poor overall survival in the Proneural subclass. Within clinical samples of the TCGA Proneural subclass, high *PROM1* expression correlates inversely with *IDH1* (R132H) mutation.

3.2.2 Discussion

Prominin-1 has been heavily debated as to its validity as a stem cell/cancer stem cell marker. Despite this I would argue that the Prominin-1 is very interesting in regards to glial cell development and differentiation. Prominin-1 is reported in multiple glial cell types; during development it has been associated with VZ regions and stem cells (Corbeil et al. 2009). In the adult it has been described in SVZ GFAP/EGFR positive stem cells but also in ependymal cells and the myelin sheaths of oligodendrocytes (Corbeil et al. 2009; Codega et al. 2014; Khatri et al. 2014).

Our results support the role of Prominin-1 as a stem/progenitor cell marker in the embryonic brain. However, during postnatal brain development (between P0-P14) there is an expression shift with decreased levels in SVZ/SGZ and *Prom1* is now found in a distributed population of *Prom1*^{hi} cells. In the mouse brain this cell population is maintained in the adult and is mainly associated with Olig2. This raises the possibility that the *Prom1*^{hi} population is in fact myelinating oligodendrocytes.

Since we can determine that *Prom1*^{hi} cells are slowly cycling or generated, due to BrdU labeling, this is a dynamic population of cells. They do not respond to mechanical lesioning of the brain as described for the NG2^{+} cell and SVZ stem/progenitor cells (Buffo et al. 2005; Dimou et al. 2008), nor did irradiation ablate *Prom1*^{hi} cells. Cycling NG2/Olig2 positive oligodendrocyte progenitors are abundant in the adult mouse brain but the low number of BrdU labeled *Prom1*^{hi} cells and lack of co-staining for NG2 distinguishes them from this population. However, the possibility remains that NG2^{+} cells are related to these *Prom1*^{hi} cells due to the Olig2 relation. NG2^{+} progenitor cells are dependent on Olig2 functions and are ablated in Olig1/2 knock-out mice, which subsequently results in lack of oligodendrocytes and embryonic lethality (Ligon et al. 2006). This is not the case for *Prom1*^{hi} cells, we also identify a subpopulation of *Prom1*^{hi} cells expressing Sox2. This suggests that they are not typical oligodendroglial cells.

Even though we detected lower *Prom1* expression in the SVZ compared to that of *Prom1*^{hi} it does not oppose the role of Prom1 in stem cells of the adult mouse SVZ, there could simply be a lower level expressed in these cells compared to the *Prom1*^{hi} cells. Neither do our results rule out the possibility that *Prom1*^{hi} cells are quiescent non-cycling stem cells that retain the capability to re-enter the cell cycle and self renew which has been described for *Prom1* expressing cells in the SVZ (Codega et al. 2014; Khatri et al. 2014). As it has been reported that there might be a distinction in the splice variants between oligodendrocytes, expressing s.3, and embryonic stem cells expressing s.1 (Corbeil et al. 2009), it would be interesting to investigate possible similarities in the adult brain. This could lead to further distinction between the *Prom1*^{hi}, Prom1 SVZ, Olig2 and Sox2 subpopulations.

The *Prom1*^{hi} cells appear to be identical to cells previously identified as neural stem cells in the early postnatal cerebellar white matter (Lee et al. 2005), but highlights that they are present throughout the entire CNS also in the adult brain. The question still remains however if those were real stem cells due to the fact that they were isolated using FACS and then assumed to be stem cells because the end result was cells from different lineages. I do not believe FACS sorting to be specific enough to make such a conclusion. Another issue is that whole cerebellum was used that could possibly hold Prom1^{+} SVZ stem cells in the developing fourth ventricle.

In the human we could not follow the developing brain to the same extent as in the mouse. We do find PROM1 expression in the ventricular zone and NSC cultures of the embryonic human brain. NSC cultures show a homogenous staining pattern of PROM1 mRNA and protein making it very hard to associate with specific lineage markers. In the adult we again

find a distributed cell population expressing PROM1, but in the human brain it is associated with GFAP and SOX2 rather than OLIG2. Here there is also a presence of PROM1 cells in the SVZ that co-localize with the stem cell marker GFAPδ. As in the mice PROM1 is detected on the ependymal cells apical surfaces and microvilli in humans.

Our results regarding PROM1 in glioblastoma further indicated that it should not be referred to as a marker expressed in only a minor subpopulation of cancer stem cells, as previously suggested (Singh et al. 2003; Singh et al. 2004). In our glioblastoma PDCL/PDX models we find varying levels of PROM1, from very high homogenous to low single cell expression. PROM1 again co-labels with SOX2 and GFAP but now to higher extent also with OLIG2. PROM1 association to GFAP, OLIG2 and Ki67 was found to be the complete opposite in different PDCLs. Using TCGA glioblastoma patient and BT-line expression data we did not detect a strong association of PROM1 with other stem cells genes. This included use of methods for RNA expression profiling, RISH, and IHC. This makes it hard to assign PROM1 cells to a certain cell state and suggest that PROM1 is tied to only cancer stem cells.

However, independent of its relevance as a CSC marker, PROM1 seems to be associated with a clinically relevant biology in glioblastoma. Multiple studies have shown PROM1 antigen or *PROM1* mRNA expression to be associated with shorter survival in patients (Beier et al. 2007; Zeppernick et al. 2008; Laks et al. 2009). In the Proneural expression subgroup *PROM1* is found to be associated with shorter survival. Examining TCGA clinical samples we find that *PROM1* expression is anti-correlated with *IDH1* (R132H) mutation, which supports a different biology for *IDH1* mutant tumors versus glioblastomas with wild type *IDH1*. This anti correlation with *IDH1* mutation would also explain the worse prognosis of high *PROM1* in glioblastomas.

The role of Prominin-1 as a stem cell marker continues to elude us. We offer several insights into the glial nature of Prominin-1 cells and discover that there are key differences in human and murine systems. Prominin-1 plays a role in glioblastoma biology but what that exact role is remains to be determined.

3.3 PAPER III

Loss of nucleolar histone chaperone NPM1 triggers rearrangement of heterochromatin and synergizes with a deficiency in DNA methyltransferase DNMT3A to drive ribosomal DNA transcription

3.3.1 Results

NPM1 depleted cancer cells and normal fibroblasts displayed deformed nucleoli and a rearrangement of perinucleolar heterochromatin. We found that NPM1 associated with HP1γ and is required for perinucleolar tethering of HP1γ foci. NPM1 is enriched, and juxtaposed, to heterochromatin in the nucleolar periphery marked by H3K9me3/HP1γ. In addition to HP1γ we found NPM1 to interact with the core histones and linker histone H1.5. Depletion of NPM1 displayed a modest decrease in H3K9 di- and tri-methylation at the rDNA promoter

but not at total cellular levels. Despite the apparent changes in nucleolar morphology there was no decrease in rDNA transcription. Neither did depletion of NPM1 cause p53 activation instead it partially abrogated the p53-dependent increase in p21, WIG1 and PUMA in response to actinomycin D or 5-FU treatment in U2OS cells. Along with these results cell proliferation was sustained in cancer cells and only slightly decreased in normal fibroblasts after NPM1 depletion. Furthermore, co-depletion of both DNA methyltransferase DNMT3A and NPM1 enhanced rDNA transcription, this was accompanied by a normalization of nucleolar morphology.

3.3.2 Discussion

NPM1 is a nucleolar chaperon protein involved in a number of processes from nucleolar stress, ribosome biogenesis to genome stability, all relevant to cancer (Grisendi et al. 2006). As we described here NPM1 also has a role in maintaining the general structural organization of the nucleolus and its surrounding chromatin. Even partial reduction of NPM1 levels resulted in a distorted nucleolar structure accompanied by perinucleolar heterochromatin rearrangement. Despite this we observed no major effect on rDNA transcription after depletion of NPM1 in cancer cells, which is perhaps logical since one can generate mouse embryonic fibroblasts (MEFs) that lack Npm1 as long as it is in combination with loss of p53. Normal human diploid fibroblasts do however show a slight reduction in cell proliferation rate after NPM1 depletion.

We could identify several NPM1 interacting proteins in glioma cell line U1242MG. Among those were several related to heterochromatin formation (H3, H1.5, HP1γ). Formation of heterochromatin is crucial for maintaining nucleolar structure and the integrity of rDNA (Guetg and Santoro 2012). This led us to further investigate the role of NPM1 in organization of the perinucleolar chromatin.

In Drosophila H1 is required for the structural integrity of heterochromatin and maintenance of pericentric heterochromatin-associated histone marks, including H3K9me2 (Lu et al. 2009), and loss of H3K9 methylation induces fragmentation of the nucleolus due to illegitimate recombination of repetitive DNA sequences (Peng and Karpen 2007). In NPM1 depleted cells we did see a reduction of H3K9 di- and tri-methylation at the rDNA promoter. However the question remains if these levels of reduction would be sufficient to alone cause the change in nucleolar morphology. We also identified HP1γ as a novel NPM1 associated protein in the perinucleolar region. NPM1 was required for efficient tethering of HP1γ to the nucleolus. HP1γ is localized to both euchromatin and heterochromatin, and is enriched in perinucleolar chromatin (Schmiedeberg et al. 2004). HP1γ is involved in maintaining normal nucleolar morphology based on its role in heterochromatin formation (Larson et al. 2012). Depletion of other proteins involved in rDNA silencing and/or heterochromatin formation such as DNMT1, HP1, SIRT1, and the histone H3K9 methyltransferase SUV39H1 also induces alterations in the nucleolar structure (Espada et al. 2007; Peng and Karpen 2007; Salminen and Kaarniranta 2009; Horakova et al. 2010). Thus, it is plausible that the effects we see after NPM1 depletion is due to a similar inability to maintain ordered heterochromatin

structure. Since NPM1 interacts with multiple proteins involved in this it may also here act as a type of chaperone, assisting in protein shuttling as well as further promoting heterochromatin stability.

A decrease in rDNA methylation results in abnormal rDNA transcription and disorganized nucleoli (Espada et al. 2007). Similarly, depletion of ribosomal proteins affects ribosomal biogenesis and nucleolar chromatin organization (O'Donohue et al. 2010). However, NPM1 was not essential for rDNA transcription in the U1242MG cell line, this is also observed in other cell lines (Colombo et al. 2005; Maggi et al. 2008), nor is NPM1 localized to the fibrillarin rich regions of nucleoli where active rDNA transcription takes place. Despite not directly affecting rDNA transcription absence of NPM1 may create a permissive environment allowing for additional epigenetic changes. Mutations in DNMT3A and NPM1 are often co-occurring in AML (TCGA 2013). Loss of DNMT1/3B function results in decreased rDNA methylation followed by increased abnormal transcription of rDNA copies and disorganized nucleoli (Majumder et al. 2006; Espada et al. 2007). Here we could show that co-depletion of NPM1 and DNMT3A synergized to enhance rDNA transcription. This would fit with our previous results suggesting a decrease in heterochromatin stability and reduction of H3K9 methylation already induced by NPM1 depletion.

We propose that NPM1 plays a crucial role in the structural integrity of the nucleolus. This is possibly due to NPM1 being involved in many of the processes concerning heterochromatin formation and its organization around the nucleolus.

3.4 PAPER IV

Expression and cellular localization patterns of the histone chaperone NPM1/nucleophosmin in glioma

3.4.1 Results

In this study, NPM1 was detected by immunohistochemistry in a cohort of human astrocytic gliomas of different grades. NPM1 was expressed in all tumors with the highest staining intensity in grade IV tumor samples. NPM1 was localized to both the nucleolus and the nucleoplasm in grade IV tumors, a distribution also seen in glioblastoma cell lines. Lower grade tumors predominantly presented with nucleolar staining, however with enlarged nucleoli. We demonstrate that NPM1 is required to maintain the structure of the nucleolus in glioma cells, confirming the role of NPM1 in nucleolar morphology described in our previous study. Depletion of NPM1 had modest effects on the viability and proliferation rate of U251MG, U1242MG, and U343MGa Cl2:6 glioblastoma cell lines. When cells were exposed to chemotherapeutic agents following NPM1 depletion we found that U251MG cells were sensitized to cell death induced by temozolomide (TMZ) and 5-Fluorouracil (5FU). Previously we identified linker histone H1.5 as an NPM1 associated protein in glioma cells. Here we demonstrate that depletion of NPM1 also sensitized glioma cells to reduced levels of H1.5 leading to the increased detection of cleaved caspase-3 (CC3) and cell death.

3.4.2 Discussion

Since NPM1 is abundant in solid tumors it was of interest for us to determine the expression patterns and explore the functions of NPM1 in gliomas. The role of NPM1 in cancer remains complex with both growth promoting and tumor suppressive functions ascribed to NPM1 (Grisendi et al. 2006). Our findings of high NPM1 levels in astrocytic gliomas suggest that NPM1 may suppress apoptosis or play some other significant pro-survival role in gliomas. Further, the increase seen in glioblastomas might implicate a role in tumor progression.

NPM1 interacts with a number of different proteins that may be involved in the organization of chromatin such as core histones and linker histone H1.5, shown by us and others (Park et al. 2010; Gadad et al. 2011; Holmberg Olausson et al. 2014). Linker histone H1 isoforms are best known for their ability to stabilize condensed higher-order chromatin structures and H1 has been shown to interact with a large network of nucleolar proteins (Kalashnikova et al. 2013). NPM1 and H1.5 interactions have been shown by us and others (Gadad et al. 2011; Holmberg Olausson et al. 2014). H1.5 depletion causes upregulation of genes involved in cell death/apoptosis and reduced cell growth (Li et al. 2012). We found that NPM1 reduction sensitized glioma cells to cell death triggered by a deficiency in H1.5, further linking NPM1and H1.5 function.

Depletion of NPM1 sensitizes U87MG and A172 glioma cells to TMZ (Gimenez et al. 2012). This was confirmed by us in U251MG cells where NPM1 depletion resulted in sensitization to TMZ and 5-FU. However the same was not as apparent for U1242MG and U343MGa Cl2:6 cells, although a similar trend was observed. The different glioblastoma cell lines had similar initial sensitivity to TMZ and 5-FU, except U343MGa Cl2:6 that was less sensitive to 5-FU, so it would appear that the increase in sensitivity seen in U251 is dependant on the loss of NPM1.

While our results demonstrate a link between NPM1 and linker histone H1.5 in the regulation of cell death it remains to be determined how this occurs. Since NPM1 depletion sensitizes glioma cells to TMZ/5FU it might be suggested that this is due more to accumulated targeting of DNA integrity rather than a specific loss of function as a consequence of lost NPM1/H1.5 interaction, however this remains to be determined. Strongest effect was seen in U251MG cells which appear to have increased aneuploidy compared to U1242MG and U343MGa Cl2:6. Hence, it would be interesting to test more cell lines where this features could be compared in relation to NPM1. NPM1 is relevant for DNA integrity and centrosome function, which could be a greater issue in cancer cells with increased aneuploidy.

Naturally one would like to investigate these effects on more cell lines including glioma cells represented by PDCLs cultured under NSC conditions. The MG-lines used in these experiments are very resistant to TMZ and 5-FU, forcing us to use very high concentrations in order to see effects. In addition we would seek to further describe the events/pathways resulting in cell death upon NPM1/H1.5 co-depletion.

In summary, NPM1 was detected in astrocytic gliomas of all grades with glioblastomas displaying the highest levels. Glioblastomas had both nucleolar and nucleoplasmic localization of NPM1 also seen in glioblastoma cell lines, in contrast to the predominant nucleolar staining in lower grade tumors. Although our studies would suggest little effectiveness of targeting NPM1 alone there could be potential in using this as a combination treatment.

4 CONCLUSIONS AND PERSPECTIVES

Glioblastoma is a truly devastating disease with short survival and no cure despite multidisciplinary treatment regimes. To make matters worse the radiotherapy and chemotherapy regimens practiced, despite the good intention of aggressively treating the tumor, in the end reduces patient quality of life with little benefit in survival (Malmstrom et al. 2012). There can also be adverse effects of chemo/radiotherapy with an induction of mutations, which might result in increased malignancy (TCGA 2008; van Thuijl et al. 2015). The progress in patient survival seen during the last decades appear to be due to better surgery and post surgery care rather than new molecular biology strategies devised for glioblastoma treatment. Current high throughput molecular screens have done little more than reconfirm known genetic events already described for glioblastoma. However, there are also new mutations and molecular pathways revealed which may help develop new treatments. The popularity boom of cancer stem cells resurfaced the old idea that cancer biology can be compared to developmental biology. Even if there still are no reliable cancer stem cell markers for glioblastoma it has had a positive influence on how we now view and (try to) understand developmental/differentiation pathways and their roles in cancer. The latest in cancer is heterogeneity, like cancer stem cells this is an old theory that gained new interest. This is a key feature of glioblastoma biology where there is plenty of evidence of a very heterogeneous nature. Unfortunately our new growing knowledge base holds no real clinical value for today's glioblastoma patients. We are now better at stratifying patients using *IDH1* mutation status. But that seems to be about it, and it doesn't really do much difference in the end.

In this thesis we first address one important shortcoming in the glioma (and whole cancer) field, the lack of fully representative cancer cell models. Earlier research has spent much time on cell lines unable to fully represent glioblastomas. Our work describes the generation of a PDCL/PDX library with great potential for further preclinical drug development.

In the second paper we further elucidate the complex expression patterns of Prominin-1 in the developing/adult brain and glioblastoma. Prominin-1 is perhaps one of the cancer stem cell markers that have gotten most publicity. Cancer stem cells (CSCs) remain a controversial field and our study, like many others, has done little to make it less so. Detecting it in both stem and differentiated cells we highlight the very context dependent relationship between Prominin-1 and cell state. To make matters even more complicated we find that Prominin-1 associates with different cell lineage markers in mice and humans. This is perhaps nothing

new in regards to glial cell biology, but serves as a reminder for us and others in the field. CSCs have been described as an almost mythical cell population responsible for tumor long-term growth and recurrence after treatment. Only as few as 5-10% of the tumor cells are said to be CSCs but in reality… no one knows.

In the third and fourth study we identify a high abundance of the nucleolar chaperone NPM1 in glioma cells. Depletion of NPM1 destabilizes the nucleolus and potentiates the effect of current treatments available for glioblastoma. Our results here are interesting, for suggesting NPM1 as a possible drug target for glioblastoma treatment but also for implying a role for the nucleolus in glioma biology. The nucleolus is our ribosome factory and the concept of "nucleolar stress" is bound to play interesting roles in cancer.

All in all, there is no easy solution when it comes to glioblastoma. With increased knowledge of the complex biology behind the disease new treatment possibilities will arise. Our work, as others, highlights the very diverse features deregulated in a single cancer type. This is where I believe today's research falls short, in addressing the plasticity of cancer cells. Cancer is a true evolutionary survivor and our limited understanding of cancer biology makes it very hard for us to design new treatments. I rather propose a "blind casting technique", concentrating efforts at constructing relevant cancer models, such as PDCLs/PDXs, and start testing all possible drugs available hopefully leaving us with something that actually works.

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